MICRODIALYSIS – A NOVEL METHOD TO STUDY PLANT-AVAILABLE NITROGEN SUPPLY IN BOREAL FOREST SOIL

Päivi Soronen
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University of Helsinki
Department of Agricultural Sciences
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HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Tiedekunta/Osasto Fakultet/Sektion – Faculty Faculty of Agriculture and Forestry		Laitos/Institution– Department Department of Agricultural Sciences
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Tiivistelmä/Referat – Abstract

Nitrogen (N) availability often limits plant growth in the boreal forest ecosystem. There has been a lack of reliable method to study soil N supply as in traditionally used potassium chloride (KCl) extraction sampling and sample preparation disturb soil structure and stimulate N mineralization, leading to the overestimation of inorganic N forms ammonium (NH_4^+) and nitrate (NO_3^-) and underestimation of organic N forms such as amino acids. Diffusion-based microdialysis technique for the sampling of soil diffusive N fluxes gives an opportunity to study soil N supply at a scale that is relevant for plant N uptake, as microdialysis probe has a membrane that reminds the plant fine root in its scale and also, to some extent, in its function. During sampling, the movement of water inside the microdialysis probe induces diffusive flux of solutes across the membrane surface along the concentration gradient. The aim of this study was to test the performance of microdialysis technique at different soil moisture content levels and its capability to monitor temporal changes in diffusive N fluxes in laboratory experiments (*ex situ*). Soil fine-scale N dynamics were further studied by comparing the diffusive N fluxes in the field (*in situ*) in boreal forest soil to multiple factors that are thought to affect forest soil N availability.

In this study, soil diffusive NH_4^+, NO_3^- and amino acid N fluxes were sampled *ex situ* from sieved soils taken from three different sites – clear-cut, spruce stand (MT spruce) and pine stand (VT pine) in Lapinjärvi, Finland in November 2017. In *ex situ* microdialysis experiments, the diffusive N fluxes were observed at three different soil moisture content levels and after N addition. *In situ* microdialysis sampling was run at the logging residue experiment of the Lapinjärvi clear-cut site and at the MT spruce site in June 2018 and at the pine logging residue experiment in Kiikala, Finland in September 2018. The results from the *in situ* microdialysis were compared with soil moisture content, pH, C-to-N ratio and temperature as well as with the net N mineralization and net nitrification rates, microbial biomass C and N contents and the concentrations of volatile monoterpenes and condensed tannins, factors that are assumed to affect N availability in forest soil.

Nitrogen fluxes sampled *ex situ* showed that the total amino acid flux in the soil taken from the clear-cut site was only half of that in the MT spruce soil whereas NO_3^- flux was two times higher at the clear-cut site than at the MT spruce site. MT spruce soil with a moisture content of 60 % water-holding capacity (WHC) had significantly higher NH_4^+ flux than the same soil in its field moisture content (44 % WHC). Nitrogen pulse was detected in all soil samples as increased NH_4^+ flux after the N addition, followed by a subsequent decrease near to the initial level. *In situ* microdialysis sampling showed that the total amino acid fluxes were 5–15 nmol N cm^{-2} h^{-1} and they dominated the total diffusive N fluxes in Lapinjärvi and Kiikala. On average, the smallest share of the total free amino acids (54 %) was observed at the control plots of the logging residue experiment in Lapinjärvi. No correlation between the KCl-extractable NH_4^+-N concentration and the diffusive NH_4^+ flux was found, but instead the KCl-extractable NH_4^+-N concentration showed a significant positive correlation with the diffusive fluxes of both total free amino acid N and nitrate. Moreover, the diffusive NH_4^+ flux correlated positively with the net N mineralization rate.

In general, *ex situ* microdialysis sampling showed 2–10 times higher amino acid fluxes and 10–20 times higher ammonium fluxes than the *in situ* microdialysis that reflects the effect of sampling, sample storage and preparation. The effect of soil moisture on the diffusive N fluxes could be further studied in laboratory experiments and *in situ*. The results of this study showed that the diffusive fluxes of different N forms are decoupled from the bulk soil concentrations. Moreover, microdialysis could be possibly used to quantify the transformation processes of N compounds in soil. These results increase the evidence that microdialysis has potential to detect temporal changes in N fluxes and possibly give new information about the ongoing processes at soil microsites.

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microdialysis, nitrogen availability, diffusive flux, ammonium, nitrate, amino acids, boreal forest soil, logging residue

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HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

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Tiivistelmä/Referat – Abstract

Typen (N) saatavuus on usein kasvien kasvua rajoittava tekijä boreaalisessa metsäekosysteemissä. Typen saatavuuden tutkimiseen ei ole ollut luotettavaa menetelmää, sillä perinteisesti käytetyssä kaliumkloridi (KCl) -uutossa maanäytteen otto ja esikäsittely häiritsevät maan rakennetta ja kiihdyttävät typen mineralisaatiota, jolloin epäorgaanisen typen, eli ammoniumin (NH_4^+) ja nitraatin (NO_3^-), osuus korostuu, ja orgaanisten typpiyhdisteiden, kuten aminohappojen osuus tulee aliarvioiduksi. Typen diffuusiovuota maassa voidaan tutkia mikrodialyysillä, joka on diffuusioon perustuva maanäytteenottotekniikka. Mikrodialyysilaitteiston keräin muistuttaa kasvin hienojuurta mittakaavaltaan ja osittain myös toiminnaltaan mahdollistaen typen saatavuuden tutkimisen mittakaavassa, jolla on merkitystä kasvien typenoton kannalta. Näytteenoton aikana veden virtaus keräimen sisällä aikaansaa liuenneiden aineiden diffuusiovuon membraanin pinnan läpi konsentraatiogradientin mukaisesti. Tämän tutkimuksen tavoitteena oli testata mikrodialyysitekniikan toimivuutta maan eri vesipitoisuuksilla ja sen kykyä seurata ajallisia muutoksia typen diffuusiovuossa laboratoriokokein (*ex situ*). Maaperän typpidynamiikkaa tutkittiin lisäksi vertaamalla kenttämittauksissa (*in situ*) boreaalisessa metsämaassa havaittua typen diffuusiovuota useisiin tekijöihin, joiden ajatellaan vaikuttavan typen saatavuuteen maassa.

Tässä tutkimuksessa NH_4^+-, NO_3^-- ja aminohappotypen diffuusiovuota tutkittiin *ex situ* seulotuista maanäytteistä, jotka oli otettu kolmelta eri kasvupaikalta marraskuussa 2017 Lapinjärveltä: avohakkuualalta (clear-cut) ja sen viereisestä kuusikosta (MT spruce), sekä männiköstä (VT pine). *Ex situ* mikrodialyysikokeissa typen diffuusiovuota maanäytteessä havainnoitiin kolmella eri kosteustasolla sekä typpilisäyksen jälkeen. Typen diffuusiovuota tutkittiin *in situ* hakkuutähdekokeella Lapinjärven avohakkuualalla ja viereisessä kuusikossa kesäkuussa 2018 sekä männyn hakkuutähdekokeella Kiikalassa syyskuussa 2018. *In situ* mikrodialyysin tuloksia verrattiin maan kosteuteen, pH-arvoon, C/N-suhteeseen, lämpötilaan sekä typen nettomineralisaatio- ja nettonitrifikaationopeuteen, mikrobibiomassan C- ja N-pitoisuuksiin sekä haihtuvien monoterpeenien ja kondensoituneiden tanniinien pitoisuuksiin, tekijöihin, joiden oletetaan vaikuttavan typen saatavuuteen metsämaassa.

Avohakkuualalla totaaliaminohappojen vuo oli ainoastaan noin puolet siitä, mitä se oli viereisessä kuusikossa, kun taas NO_3^--vuo oli avohakkuualalla kaksinkertainen verrattuna kuusikkoon. Metsämaan NH_4^+-vuo oli kolminkertainen kosteuden ollessa 60 % vedenpidätyskapasiteetista (WHC, *water-holding capacity*) verrattuna NH_4^+-vuohon kenttäkosteudessa (44 % WHC). Typpilisäys puolestaan havaittiin kaikissa maanäytteissä kohonneena NH_4^+-vuona lisäyksen jälkeen, mitä seurasi vuon lasku alkuperäiselle tasolleen. *In situ* mikrodialyysin tuloksissa totaaliaminohappojen vuo oli 5–15 nmol N cm^{-2} h^{-1}, ja niiden osuus kokonaistypen vuosta oli keskimääräisesti suurin sekä Lapinjärvellä että Kiikalassa. Pienin keskimääräinen aminohappotypen osuus (54 %) kokonaisvuosta havaittiin Lapinjärven hakkuutähdekokeen kontrollialoilla. KCl-uuttuvan NH_4^+-typen pitoisuuden ja ammoniumin diffuusiovuon välillä ei ollut korrelaatiota, mutta sen sijaan KCl-uuttuvan NH_4^+-typen pitoisuus korreloi positiivisesti sekä totaaliaminohappojen että nitraatin diffuusiovuon kanssa. Lisäksi ammoniumin diffuusiovuo korreloi positiivisesti typen nettomineralisaationopeuden kanssa.

Yleiseti ottaen *ex situ* mikrodialyysinäytteenotoissa saatiin 2–10 kertaa korkeampia aminohappojen diffuusiovuon ja 10–20 kertaa korkeampia ammoniumin diffuusiovuon arvoja kuin *in situ* mikrodialyysillä, mikä heijastaa näytteenoton, säilytyksen ja esikäsittelyn vaikutuksia. Maan kosteuden vaikutusta typen diffuusiovuohon voitaisiin tutkia useammilla kosteustasoilla laboratoriossa ja kentällä. Tämän tutkimuksen tulokset osoittavat, että eri typpimuotojen diffuusiovuot eivät ole kytköksissä niiden pitoisuuteen maassa. Lisäksi mikrodialyysiä voitaisiin mahdollisesti käyttää typpiyhdisteiden muuntumisprosessien seurantaan ja kvantifiointiin lähes häiriintymättömästä maasta. Nämä tulokset vahvistavat aiempaa käsitystä siitä, että mikrodialyysillä pystytään havaitsemaan ajallisia muutoksia typen vuossa ja mahdollisesti saamaan uutta tietoa käynnissä olevista prosesseista maassa.

Avainsanat – Nyckelord – Keywords
mikrodialyysi, typen saatavuus, diffuusiovuo, ammonium, nitraatti, aminohapot, metsämaa, hakkuutähde

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1 INTRODUCTION

Nitrogen (N) is a primary macronutrient and its availability often limits plant growth in the boreal forest ecosystems. It is a component of proteins, enzymes, phospholipids and nucleic acids, and therefore plants need N in large quantities (Chapin et al. 2011, pp. 229-231). Inorganic and organic N compounds are released in soil *via* decomposition of N-containing detritus by soil fauna and microbes. Inorganic N forms that may occur in soil are ammonium (NH_4^+), nitrate (NO_3^-) and nitrite (NO_2^-) while soil organic N compounds include for example proteins, peptides, amino acids, nucleic acids and amino sugars. According to the classical paradigm, mineralization of the soil organic N into inorganic N by soil microbes is the critical step in N cycle and plants can use only inorganic N forms, mainly the leftovers that are not used by microbes themselves. New paradigm, emerged in 1990s, suggests that instead of mineralization, the rate-limiting step of N cycle is depolymerization of the organic N-containing polymers into organic N-containing monomers such as amino acids (Schimel & Bennett 2004). Moreover, it has been shown that many plant species, including several forest trees can directly utilize amino acids as N source (Näsholm et al. 1998).

Plants acquire nitrogen from soil *via* three pathways: diffusion, mass flow and root interception. Nitrogen uptake by plant roots requires that the plant-available N compounds are released from the soil solid phase into the soil solution and moved to the root surface where they can be absorbed by the live root cells (Comerford 2005). Diffusion might be the main mechanism for plant N acquisition in N-limited environments, especially in conditions where transpiration is decreased and subsequently, mass flow reduced (Comerford 2005). Diffusive N fluxes in soil can be examined by using microdialysis (Inselsbacher et al. 2011), a diffusion-based sampling and sample preparation method that has been utilized in neuroscience and pharmacokinetic studies for decades (Delgado et al. 1972, Benveniste et al. 1989) and introduced for environmental research more recently (Miró & Frenzel 2004).

Inselsbacher and Näsholm (2012a) suggested the use of microdialysis as a method to study soil N dynamics *in situ*. Traditionally used water and 1 M potassium chloride

(KCl) extraction methods are ambiguous since they require preparation procedures such as soil sieving that can severely alter not only the soil structure but also the chemical composition of the soil sample (Inselsbacher 2014). In recent studies, microdialysis has been increasingly used as an *in situ* soil sampling technique to investigate for example the potentially important role of amino acids for plant N nutrition in the boreal forests (Inselsbacher & Näsholm 2012a) and the effect of fertilization on the diffusive N fluxes (Inselsbacher et al. 2014). The relationship between the plant N uptake capacity and the diffusive N fluxes has been examined as well (Oyewole et al. 2016). It has been recognized that due to their microscale size and performance as "a sink of free moving compounds" (Inselsbacher et al. 2011), the microdialysis probes might, to some extent, mimic plant uptake of nitrogen compounds *via* diffusion when water is used as a perfusion fluid (Inselsbacher & Näsholm 2012a). On the contrary to the results obtained from the 1 M KCl and water extractions, it has been shown that amino acids might dominate soil N supply and even be the major source of N for plants in the boreal forests (Inselsbacher & Näsholm 2012a, Oyewole et al. 2016).

This thesis was done as part of a project "Measuring plant-available nitrogen in forest soil", funded by Marjatta ja Eino Kollin säätiö, in Natural Resources Institute Finland (Luke). The project was led by Docent Aino Smolander in Luke and, in addition to Luke's staff, it collaborated with Prof. Torgny Näsholm's research group (Swedish University of Agricultural Sciences, SLU, Umeå, Sweden). The purpose of this thesis was to establish a practical combination of the microdialysis sampling method and sensitive analysis methods for the determination of different plant-available N pools from a small sample volume. The performance of microdialysis as a minimally disturbing *in situ* sampling technique to study the soil N availability was evaluated and the results were compared with the parameters that are supposed to affect N availability in forest soils. The results of this study, especially from the *in situ* microdialysis sampling, will be further utilized in planning of new studies regarding for example the comparison between the effects of slow- and fast-release N fertilizers on soil N availability.

2 NITROGEN CYCLE IN BOREAL FOREST ECOSYSTEM

Nitrogen is a component of life-sustaining macromolecules such as proteins and deoxyribonucleic acids (DNA). It is also an essential component of chlorophyll. Nitrogen-containing compounds can be divided into two groups: inorganic and organic. Inorganic N-containing compounds that can be found in soil are ammonium, nitrate and nitrite. Nitrite concentration is, however, generally low in most soils (Chapin et al. 2011, p. 277). Organic N-containing compounds that occur in soil are proteins, peptides, amino acids, nucleic acids and amino sugars. Proteins, peptides and amino acids form a group of proteinaceous N compounds that are the most abundant N compounds in soil (Schulten & Schnitzer 1998). Nucleic acids, that include deoxyribonucleic acids and ribonucleic acids (RNA) as major classes, are a group of genetic information -carrying macromolecules that govern protein synthesis in the cells of living organisms. Amino sugars, in turn, are a group of monosaccharide derivatives where an amino group ($-NH_2$) has replaced one of the hydroxyl groups of the monosaccharide unit.

Nitrogen cycle in boreal forest ecosystem (Figure 1) includes additions, transformations, transport processes and losses. Nitrogen may be added to forest ecosystem *via* three different pathways: biological N fixation, deposition and fertilization. Biological N fixation is a process where atmospheric nitrogen gas (N_2) that contributes to approximately 78 % of the total volume of atmosphere, is fixed by N-fixing bacteria into bioavailable form. Nitrogen-fixing bacteria can break down the triple bond of atmospheric N_2 and reduce it to NH_4^+ using nitrogenase enzyme as a catalyst (Chapin et al. 2011, p. 267). There are different types of N-fixing bacteria; associative bacteria that are either nodulated (symbiotic) such as *Rhizobium* and *Frankia* or non-nodulated such as *Azotobacter* and *Bacillus*, and free-living (Chapin et al. 2011, p. 267). Deposition of N is, at least to some extent, a source of N input in all ecosystems. Nitrogen may be deposited in three different forms in ecosystems; in particulate form, dissolved in solution or in gaseous form (Chapin et al. 2011, p. 269). In southern parts of Finland, the mean annual input of N *via* deposition was approximately 3.0–4.4 kg ha^{-1} in 2006–2010 (Salemaa et al. 2019). Fertilization with commercial N fertilizers, which are generally produced by fixing atmospheric N_2 chemically in plant-available form in Haber-Bosch process, is an anthropogenic N input

to the ecosystem. In Finland, forest fertilization increased rapidly in 1960s, but it ceased almost entirely in 1990s because of the economic depression, change in forestry, termination of the subsidies and the uncertainty of environmental impacts (Saarsalmi & Mälkönen 2001). Overall, N additions *via* deposition or fixation in terrestrial ecosystems are of less importance than the N input *via* decomposition of organic matter since more than 90 % of N originates from this internal recycling process (Chapin et al. 2011, p. 238).

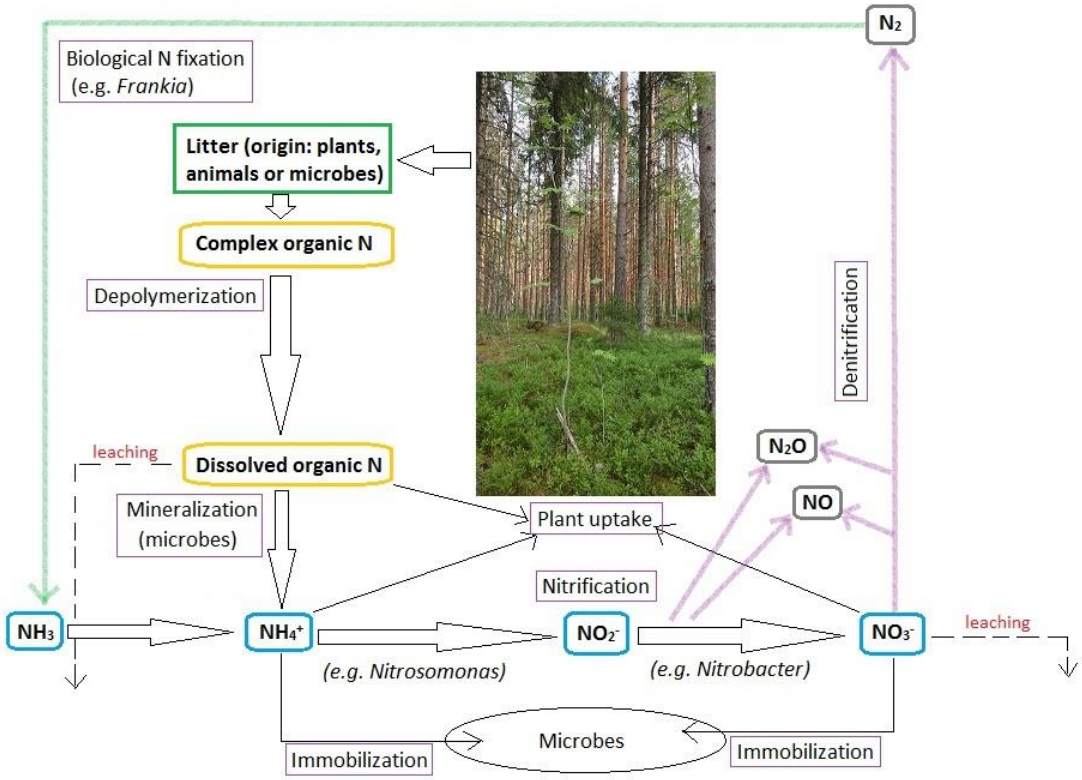

Figure 1. Schematic illustration of nitrogen (N) cycle in boreal forest ecosystem. Boxes represent organic (orange) and inorganic (turquoise) N forms in soil and the gaseous N forms (grey) in atmosphere. The rectangular, purple boxes show the processes related to N cycle.

In addition to the biological N fixation, there are several types of transformation processes included in natural, tightly closed N cycle. Nitrogen-containing litter is chewed into smaller debris and further decomposed by soil fauna and microbes. *Depolymerization* is a process where a polymer is converted into a monomer or a group

of monomers by exoenzymes that are extracellular enzymes functioning outside of the cell from where they are secreted. Complex organic N compounds can be converted into organic monomers such as amino acids in this process. Organic matter is turned over by soil microbes and converted into inorganic substances such as NH_4^+ in a process called *mineralization*. Studies from recent decades have shown that depolymerization, instead of mineralization as previously thought, might be the rate-limiting process in N cycle in N-limited forests (Schimel & Bennett 2004). Ammonium can be further utilized by microbes or plants as nitrogen source or it may be adsorbed onto the negatively charged surfaces that can occur in the soil colloidal fraction (clay and humus). In *nitrification* process, NH_4^+ is oxidized to NO_2^-, and finally to NO_3^- by nitrification bacteria. Nitrite is not usually detected in forest soils but it may sometimes accumulate in dry conditions where the activity of *Nitrobacter* is limited (Chapin et al. 2011, p. 277). Mineralization of dissolved organic nitrogen (DON) to NH_4^+ and its transformation into NO_3^- is thought slow in the boreal forest ecosystem because of the low nitrogen availability that is linked to the low litter quality (Vitousek & Howarth 1991). Moreover, nitrification is inhibited by the low pH of these soils (Paavolainen & Smolander 1998). If soil DON content is low, microbes may absorb the inorganic N forms to meet their N need and thereby compete with plants of the available inorganic N pool in a process called N *immobilization* that includes also the chemical fixation of the inorganic N forms (Chapin et al. 2011, p. 271). Microbial immobilization is an important sink of the available N in the boreal forest soils, as increased N immobilization leads to reduced availability of N for plants (Högberg et al. 2017). By subtracting N immobilization from the gross N mineralization that is the actual N mineralization, net N mineralization can be calculated. Similarly, net nitrification can be calculated by subtracting immobilization from the gross nitrification.

Nitrate is considered an inert solute in soil because it does not interact with the soil solid phase due to its negative charge. Therefore, nitrate is prone to leaching and may end up in the bodies of water where it contributes to eutrophication. Usually in undisturbed boreal forest ecosystem, nitrification is low, and nitrate is readily taken up by the growing plants. However, if the ecosystem is disturbed by for example clear-cutting, nitrate leaching may be of importance, as the vegetation is removed and consequently, nitrate uptake reduced. Ammonium and those amino acids that occur as cations in soil

solution are attracted by the negatively charged surfaces on soil particles, making them less prone to leaching compared to nitrate and those amino acids that are anions in soil solution. Another pathway for nitrate losses is a process called *denitrification* where nitrate is converted into nitric oxide (NO), nitrous oxide (N_2O) or nitrogen gas by denitrifying bacteria. Denitrification typically occurs in conditions where oxygen level is low and nitrate concentration high (Chapin et al. 2011, p. 281). Nitrification may also produce NO and N_2O as side products.

3 SOIL NITROGEN AVAILABILITY AND FACTORS AFFECTING IT

Soil nutrient bioavailability is defined as the ability of the soil-plant system to deliver essential nutrients for plants during a certain time period, resulting from the processes that regulate the release of nutrients from the soil solid phase, their movement in soil solution, and finally, their uptake by plant roots (Comerford 2005). The release of nutrients from the soil solid phase may include physicochemical processes such as desorption and dissolution, and biochemical processes such as mineralization (Comerford 2005). Availability of nitrogen often limits the primary production in terrestrial ecosystems (Vitousek & Howarth 1991). Even though nitrogen is abundant in the boreal forest soils, most of it is present in large recalcitrant organic compounds and only small share of it is plant-available, as boreal forests have typically low microbial activity due to unfavorable conditions and therefore, slow decomposition rates.

3.1 Solute movement towards plant roots

Besides inorganic N forms NH_4^+ and NO_3^-, small organic compounds such as amino acids are directly available for plant uptake and some plant amino acid transporters have been identified recently (Näsholm et al. 2009). Rather than the total concentration of N in soil or the kinetics of the plant N uptake, the flux of nitrogen compounds in soil solution might be the most important factor limiting N availability for plants and thereby, the tree growth in boreal forest ecosystems (Oyewole et al. 2016). Soil N compounds may become available for plant uptake in the way of solute movement by

diffusion or mass flow, and as roots grow to the areas of higher nutrient concentration in soil by 'root interception'. Diffusion and mass flow are the two main processes that contribute to plant N acquisition, and they are controlled by the interactions between plant and soil (Nye & Tinker 1977, p. 75, Comerford 2005). Mass flow means the movement of solutes along with the soil solution that flows towards roots mainly driven by transpiration (Chapin et al. 2011, p. 239). Root interception, in turn, is of minor importance for the plant N acquisition (Chapin et al. 2011, p. 240).

Diffusion is a transport process driven by the concentration gradients of the solute in different parts of soil. As an example, active uptake of nutrients by plant roots creates concentration gradients in soil solution around them. Fick's first law (Equation 1) describes the diffusion of nutrients in soil as a function of diffusion coefficient and the concentration gradient:

$$F_D = -D\frac{\partial C}{\partial \chi}, \quad (1)$$

where
F_D = diffusive flux (g cm^{-2} s^{-1})
D = diffusion coefficient (cm^2 s^{-1})
C = concentration of nutrient (g cm^{-3} soil)
χ = distance (cm).

Minus sign indicates that the direction of the diffusive flux is down the concentration gradient ($\partial C / \partial \chi$) from higher concentration to lower. Many factors affect the rate of nutrient movement in soil. Impedance factor, as an example, is a term describing the relationship between the path length of the straightforward movement of mineral nutrient to its actual path length in soil (Tinker & Nye 2000 p. 79, Comerford 2005).

3.2 Soil physicochemical factors

Soil pH affects many processes that are supposed to control the bioavailability of nitrogen, including such physicochemical processes as sorption, desorption and

dissolution of nutrients (Comerford 2005) as well as biochemical processes like mineralization and nitrification (Paavolainen & Smolander 1998). It is also dependent on soil pH, whether the different amino acids are in anionic or cationic form and moreover, how they react with the soil particle surfaces. Diffusion of non-volatile compounds in soil is regulated by the volumetric water content (vol-%) since it determines the cross-sectional area accessible for the diffusive flux (Comerford 2005). Soil temperature affects not only the rate of organic matter decomposition and release of plant-available N but also the diffusive fluxes of N compounds.

3.3 Litter quality

Composition of litter and soil organic matter affects soil N availability. Litter C-to-N ratio has been recognized to affect the rate of decomposition (Chapin et al. 2011, p. 196). Nitrogen-rich litter that has a low C-to-N ratio is decomposed quickly (Högberg et al. 2017), especially during the initial phase of decomposition when the microbes use more easily degradable material. Decomposition rate slows down over time, as more recalcitrant compounds are left in litter (Chapin et al. 2011, p. 197). The quality of litter is affected by for example, the concentration of plant secondary compounds in it as these compounds are generally considered recalcitrant. Plant secondary compounds are a large and diverse group of compounds that are products of plant secondary metabolism. There is increasing evidence available that certain plant secondary compounds such as terpenes and phenolic compounds may control nitrogen cycling in forest soils (Smolander et al. 2012). This evidence has been obtained mostly from exposing soil samples to these compounds in the laboratory and their real significance in field conditions is still a question.

Terpenes are a complex group of plant secondary compounds that are built from various number of isoprene C5 units. Monoterpenes have two C5 units and they have been studied most from this group. They occur in almost all plants and are the most abundant group of volatile organic compounds (VOCs) emitted by coniferous trees, mainly from their needles (Guenther et al. 1994). Smolander *et al*. (2012) reviewed that volatile monoterpenes may have an inhibitory effect on net N mineralization and net nitrification, and therefore reduce the N availability in forest soils.

Tannins are a major class of plant secondary compounds that are widely distributed in vascular plants. They are water-soluble polyphenolics in chemical composition and have a molecular weight of 500–3000 Daltons (Da) (Hättenschwiler & Vitousek 2000). Production of tannins possibly developed in plants as a defense means against pathogenic bacteria and fungi, insects and herbivores as tannins can precipitate proteins and other macromolecules (Bernays et al. 1989, Gessner & Steiner 2005). Moreover, they act as antioxidants (Gessner & Steiner 2005) and UV-protecting agents and affect plant colors and the taste of food and drinks, thereby influencing not only plant biology but also human life (Hättenschwiler & Vitousek 2000). Tannins may have multiple important functions at ecosystem level since they potentially affect nutrient cycling by lowering the decomposition rates, complexing proteins, causing toxicity to microbes and reducing enzyme activities (Hättenschwiler & Vitousek 2000, Kraus et al. 2003). They can be divided into two main groups: (1) condensed tannins, also known as proanthocyanidins that are the most abundant group of polyphenolics in woody plants and (2) hydrolysable tannins, divided into gallotannins and ellagitannins, and less abundant with the occurrence restricted to approximately half of the dicotyledons (Hättenschwiler & Vitousek 2000). Despite the diversity of condensed tannins, the linear or branched polymeric structures can be derived from relatively few low-molecular weight monomers such as flavan-3-ols (Figure 2).

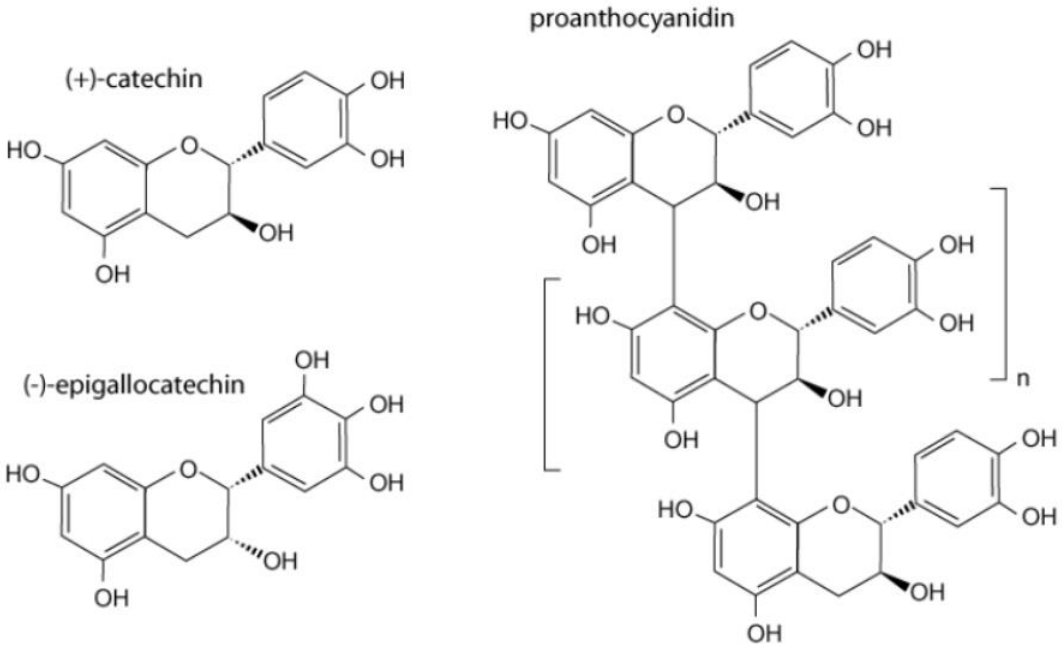

Figure 2. Molecular structures of flavan-3-ols (+)-catechin and (-)-epigallocatechin, examples of monomeric units that are connected by C4-C8 bounds to form a linear proanthocyanidin structure (Gessner & Steiner 2005).

3.4 Logging residue harvest as an example of forest management practice affecting N transformations

The natural, usually tightly closed N cycle in our forests can be disturbed by human activities such as clear-cutting. In clear-cutting, logging residues including tree tops, branches and needles, can be left on the site or harvested since they are nowadays recognized as valuable material for bioenergy. Currently logging residues are harvested mostly from final fellings (in about one third of all final fellings), and less from thinning stands. With regard to logging residue harvest, the advice is to leave approximately 30 % of the logging residues on the site. In both harvesting techniques, stem-only harvest and whole-tree harvest, logging residues are not evenly distributed but as piles on the area. Not only the nutrients are lost from the clear-cut site with the harvested biomass, but also the logging residue piles may have significant effects on the soil underneath them. In final fellings, logging residues may alter soil properties and enhance net N mineralization and net nitrification and thereby affect N availability as well as contribute to N losses (Törmänen et al. 2018). In thinning stands, logging residue

harvest has been shown to decrease net nitrogen mineralization in long-term, affect enzyme activities and change plant secondary compound composition (Smolander et al. 2008, Smolander et al. 2010, Smolander et al. 2013, Adamczyk et al. 2015).

4 MICRODIALYSIS AS A SOIL NITROGEN SAMPLING TECHNIQUE

As analytical techniques have evolved, the development of soil sampling and sample preparation methods has been slow (Miró & Frenzel 2011). Soil sieving and extraction have been recognized as potential sources of misinterpretation in soil nitrogen studies (Inselsbacher 2014), as sampling itself causes disturbance to the natural state of environment and the sample preparation may lead to further transformations and losses of the analyte as well as to increased contamination risk (Miró & Frenzel 2011). Microdialysis is a novel approach for the soil nitrogen studies that causes only minimal disturbance for the soil structure and enables the sampling of soil solution *in situ*. Moreover, it allows only low-molecular weight compounds to enter the sample, and thus excludes microbes and large organic molecules that might cause further disturbance for the chemical composition of the sample or interfere with the determination of the analyte. There is no need for further sample preparation steps, as the microdialysis itself is an effective sample cleanup technique (Miró & Frenzel 2011). Therefore, microdialysis has been recognized as a promising soil sampling and sample preparation method for the soil nitrogen studies (Inselsbacher et al. 2011).

4.1 History of microdialysis - from brains to soils

Sampling of extracellular tissue concentrations of different compounds with the microdialysis technique was introduced almost 50 years ago by Delgado *et al*. (1972). Since that, microdialysis has been extensively used in neurochemical and pharmacokinetic research (Benveniste et al. 1989, Torto et al. 2001). Mathematical framework has been developed to provide a quantitative basis for the microdialysis approach, as used for the *in vivo* tissue concentration measurements based on the concentration of the studied compound in the dialysate and for the *in vitro* probe

characterization (Bungay et al. 1990). The potential of microdialysis for the environmental monitoring was recognized more recently (Torto et al. 2001, Miró & Frenzel 2004). Inselsbacher *et al.* (2011) presented the use of microdialysis for soil sampling as a method to study soil N dynamics and since that, the method has been increasingly used for this purpose (Inselsbacher & Näsholm 2012a, Inselsbacher & Näsholm 2012b, Oyewole et al. 2014, Inselsbacher et al. 2014, Shaw et al. 2014, Oyewole et al. 2016, Oyewole et al. 2017, Leitner et al. 2017, Buckley et al. 2017, Jämtgård et al. 2018, Hill et al. 2019). Recent studies using microdialysis for the *in situ* sampling of the diffusive N fluxes have revealed that organic N, especially amino acids, might be the most important source of N for plants in the N-limited boreal forest soil (Inselsbacher & Näsholm 2012a, Oyewole et al. 2016).

4.2 Description of the technique

Microdialysis is based on passive diffusion of the target molecules across a porous membrane that is permeable to water and small solutes (Figure 3). The flow of water inside the membrane induces diffusive flux that is defined as the amount of molecules crossing the membrane surface area in unit time (expressed here as nmol cm^{-2} h^{-1}) towards lower concentration of the molecule by diffusion. The diffusive flux across the membrane surface from the surrounding environment in the intimate vicinity of the microdialysis probe is driven by the concentration gradient between the perfusion fluid and the external medium where the membrane is placed. The resulting solution called dialysate is collected into a vial and analyzed for the concentrations of the studied compounds. The relative recovery (RR) is a term used to describe the relative share (%) of the concentration of the compound in the dialysate as compared to its concentration in the external medium.

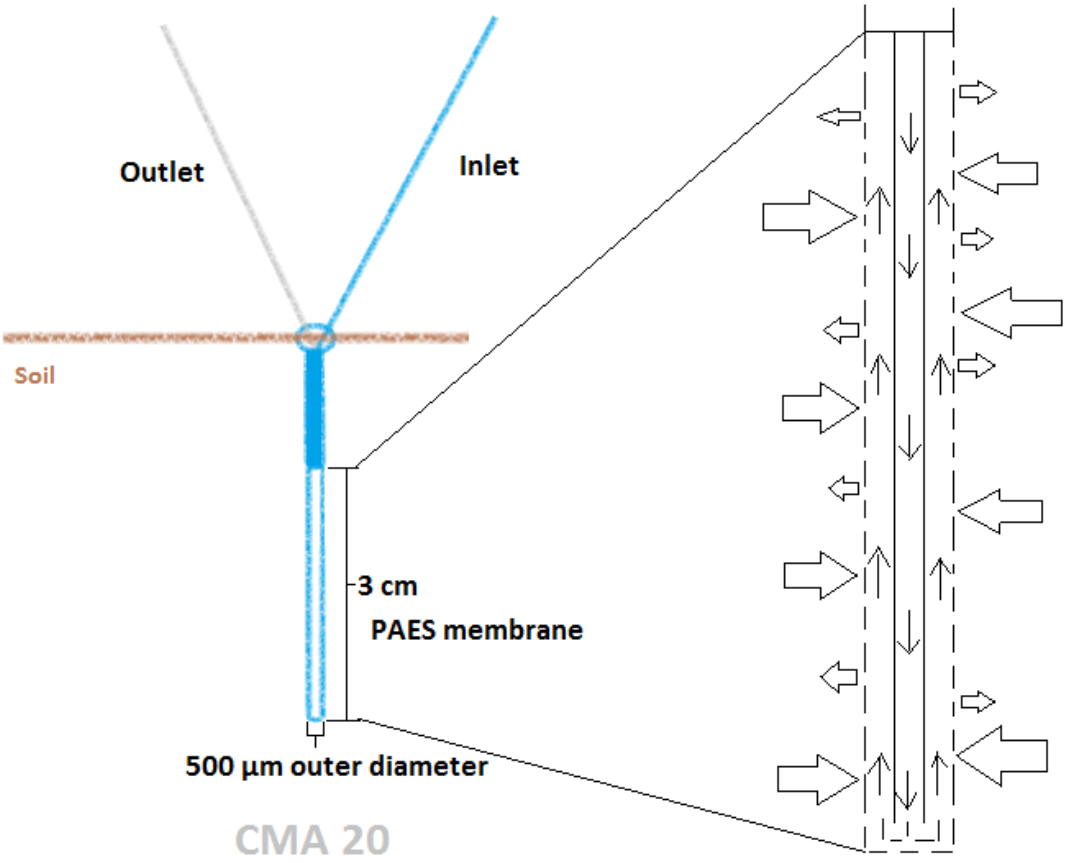

Figure 3. A schematic illustration of a microdialysis probe (CMA 20) with 30 mm membrane placed in soil. Arrows show the directions of the influx and efflux that are the fluxes of a compound into the membrane and out from the membrane to the soil, respectively. The size of the arrow represents the assumed magnitude of flux. The depicted measures of the membrane are not to scale. PAES = polyarylethersulfone. Adapted from Buckley *et al*. (2017).

The concentration of the target compound in the dialysate is dependent on the characteristics of the dialysate (d), membrane (m) and the external medium (ext). A concept of mass transfer resistance (R) is utilized in the theoretical framework for microdialysis to describe the resistances targeted to the movement of solute at the dialysate-membrane and membrane - external medium interfaces (Bungay et al. 1990). The resistance for the dialysate (R_d) is determined by the characteristics of the perfusion fluid, for example its temperature and viscosity as well as the solutes already present in it (Miró & Frenzel 2011). The membrane resistance (R_m) is determined by the properties of the membrane, such as its material and molecular weight cut-off (MWCO) that is the pore size of the membrane in Daltons that prevents 80 % of the molecules of

that size from passing the membrane surface. Diffusion of the molecules in the external medium towards the microdialysis membrane is controlled by the volume fraction accessible for diffusion in contact with the membrane and the tortuosity of the diffusion path (Benveniste et al. 1989) that together define the external medium resistance (R_{ext}). According to Bungay *et al*. (1990), R_{ext} is generally the most important factor determining the solute movement from the external medium into the membrane ($R_{ext} >> R_m >> R_d$) and it is largely dependent on the environment where the microdialysis probe is placed. While the probe is placed in a stirred solution where the concentration of solute around the probe is constantly replenished, the R_{ext} can be considered much smaller, and practically negligible, than in such a heterogeneous medium as soil.

4.3 Applications and advantages of microdialysis in soil nitrogen studies

The dialysate resistance R_d is considered negligible as compared to the R_m or R_{ext}. However, R_d can be manipulated by the choice of perfusion fluid and flow rate. The slower the flow rate, the more concentrated the dialysate will become, and this might be helpful for the analyses. However, this means also longer sampling time, that is in turn impractical especially in the field. A typical configuration of the microdialysis probe used for soil sampling has a MWCO of 20 kDa and is 10 mm long (e.g. Inselsbacher et al. 2011, Inselsbacher & Näsholm 2012a, Inselsbacher et al. 2014, Oyewole 2015). It allows low-molecular weight compounds to pass but prevents larger molecules and for example, microbes from entering the dialysate. Its disturbance to the soil environment is considered minimal due to its small size (Inselsbacher et al. 2011, Oyewole 2015). Aims at reducing R_m have been made by testing different membrane configurations with longer, 30 mm membrane or alternatively, with larger MWCO of 100 kDa (Buckley et al. 2017). It was found that the longer, 30 mm membranes would increase the relative recovery of especially the water-soluble, mobile N compounds from a stirred solution and consequently, the concentrations of the target compounds in the dialysates sampled from soil (Buckley et al. 2017). Simultaneously, the diffusive fluxes might be smaller due to the increased surface area and therefore, the comparison between the results of the soil diffusive N fluxes obtained with different microdialysis probe configurations might be rather difficult.

When the microdialysis probe is installed in the heterogeneous soil matrix, it is difficult to predict what kind of soil environment it meets. The amount of target compounds passing through the membrane surface over time (flux) is dependent on the R_{ext} of the environment where the membrane is placed (Bungay et al. 1990). When the probe is placed in soil, R_{ext} is affected by soil moisture and structure that together define the membrane surface area that is in contact with the soil solution and the tortuosity of the diffusion path. However, it can be thought that the microdialysis probe faces similar R_{ext} as plant fine root in soil, and therefore the result may represent the N availability for plants (Inselsbacher et al. 2014). It must be noted though, that the microdialysis probes lack the ability to simulate active nutrient uptake by plant roots *via* ion channels and transporters (Inselsbacher et al. 2014). Instead of absolutely mimicking the uptake of N compounds by plants, the diffusive N flux observed using microdialysis technique might therefore reflect soil plant-available N supply *via* diffusion when high-purity deionized water is used as perfusion fluid (Inselsbacher et al. 2011).

Microdialysis technique was further developed by Oyewole (2015) to measure the contribution of the mass flow flux of N compounds to the soil N supply. This was done by using a solution containing Dextran 40 as perfusion fluid to achieve a lower osmotic potential in comparison to the use of water as perfusate and thereby, induce mass flow flux of water and solutes across the membrane. It was noticed that including mass flow increased soil N fluxes significantly and altered the chemical composition of N fluxes as well. Especially in nutrient-rich ecosystems, mass flow increased the share of nitrate of the total N flux. Given the importance of transpiration for the plant nutrient uptake and the observed increase in the total N fluxes, Oyewole (2015) concluded that mass flow is important for plant N acquisition in boreal forests.

An important advantage of the microdialysis technique in soil N studies is that it enables the sampling of soil nutrient fluxes *in situ* with minimal disturbance to soil structure, an approach that has not been possible with the traditional methods. Traditionally used water and KCl extraction methods might be ambiguous since the sample preparation such as soil sieving as well as the extraction itself can alter the chemical composition of the soil sample. It has been studied that the soil extractions, especially when combined to soil sieving, may lead to increased mineralization of DON

and consequently, a potential overestimation of the inorganic N pool (Inselsbacher 2014).

4.4 Challenges faced by microdialysis approach

It is important to ensure that the performance of the microdialysis probes stays constant over the sampling period. Performance may change due to for example binding of proteins on the membrane surface, fouling or damage. In order to notice the change in performance that might have occurred during sampling, *in vitro* 'calibration' of the microdialysis probes is needed before the membrane is placed in soil and moreover, the probe calibration should be checked in between the sampling events. According to the microdialysis probe calibration procedure (Bungay et al. 1990, Inselsbacher et al. 2011), probes are calibrated by immersing them in a stirred solution containing a known concentration of each compound studied and perfusing them with deionized water at a given flow rate. However, even though important in terms of noticing the damage or fouling of the probe, there is a lack of applicability of this calibration procedure into the *in situ* probe performance. Conclusions of the concentration of a target compound in soil solution solely based on their relative recovery from a stirred solution are problematic because the R_{ext} is completely different in these two media. As Bungay et al. (1990) noted the R_{ext} approaches zero in a perfectly stirred solution. In a more complex medium, it is affected not only by the volume fraction accessible for diffusion and the tortuosity of the diffusion path (Benveniste et al. 1989) but also by the relevant physical and biochemical processes that must be considered, too (Bungay et al. 1990). For the soil N availability, relevant processes are for example the decomposition rate of the soil organic matter that replenishes the pool of plant-available N as well as the sorption-desorption reactions of the different N forms between the soil solution and the soil solid phase.

Hobbie and Hobbie (2013) noted that the installation of the microdialysis probes in soil damages fine roots and mycorrhizal hyphae, thereby overestimating the flux of certain amino acids such as glutamine and glutamic acid that are considered the most abundant amino acids in the fine roots and hyphae. Inselsbacher *et al.* (2014) studied the amino acid fluxes *in situ* boreal forest soil for an extended sampling period of 100 min with a

20-minute sampling interval to address this possible artifact of the microdialysis approach. They concluded that the damage of fine roots and hyphae is of minor influence since the amino acids dominating the diffusive flux of total free amino acids were histidine, glycine, serine and alanine, whereas the contribution of glutamine and glutamic acid was lower. Moreover, they found that the amino acid flux stayed relatively stable over the sampling period, indicating that the large share of amino acids might be an inherent characteristic of boreal forest soils.

4.5 Way forward

As soil is a heterogeneous medium, its properties can vary a lot within very small scale and microdialysis offers new insights into N dynamics at soil microsites. In future, microdialysis could be utilized to determine the need for N fertilization. This requires, however, that microdialysis is further developed to more easy-to-use method for practical purposes. Microdialysis might not only deepen the knowledge on plant nutrition and the nutrient fluxes in soil, but also give new information about environmentally relevant issues. To give one example, as a consequence of climate change, seasonal drought may become more frequent in Finland in future. In a case study by Smolander *et al*. (2005b), it was found that the soil microbial activity and consequently, net N mineralization rate decreased due to a prolonged (2 months) drought. However, the subsequent rewetting of the soil led to increased net nitrification rate. By using microdialysis, Leitner *et al.* (2017) found that drought could cause accumulation of nitrogen in soil and a subsequent leaching of mobile N forms, especially nitrate and some hydrophilic amino acids due to rewetting event such as heavy rain in temperate forests.

5 RESEARCH OBJECTIVES

The aim of this thesis was to obtain a better understanding of the soil fine-scale N dynamics by using microdialysis as a soil sampling method. As it has been discussed, soil plant-available N supply is affected by several soil physicochemical factors and

biochemical processes. The relationships between the soil diffusive N fluxes and the soil characteristics affecting N availability were investigated. The performance of the microdialysis method both in laboratory (*ex situ*) and in field (*in situ*) conditions was examined and the effect of soil properties such as moisture content, N concentration, temperature and the concentration of plant secondary compounds on soil diffusive N fluxes was observed. *In situ* microdialysis sampling was run in a logging residue experiment on a clear-cut and in adjacent spruce stand, and in a logging residue experiment in a pine thinning stand. It was hypothesized that:

(i) Clear-cut site has lower amino acid fluxes and higher nitrate fluxes, as compared to the adjacent forest.
(ii) Diffusive nitrogen fluxes increase with increasing soil moisture content.
(iii) Nitrogen addition causes a peak in soil diffusive nitrogen fluxes, but the fluxes will decrease subsequently due to immobilization, adsorption of ammonium to soil solid phase and the formation of diffusion shell around the microdialysis probe.
(iv) Spruce logging residue piles affect the rates of net N mineralization and net nitrification leading to increased diffusive fluxes of mineral nitrogen in the soil underneath.

6 MATERIALS AND METHODS

6.1 Study sites

Samples were collected from Lapinjärvi and Kiikala in 2017–2018. Three sites in Lapinjärvi were chosen for the *ex situ* microdialysis sampling and subsequent experiments in the laboratory. The first site was a clear-cut area (Figure 4a; 'clear-cut'). The second site was a Norway spruce (*Picea abies* (L.) Karst.) -dominated stand (later referred 'MT spruce') next to the clear-cut area. The floristic forest site type was MT (*Vaccinium myrtillus*) for both clear-cut and spruce stand (Cajander 1949). The third

site was a Scots pine (*Pinus sylvestris* L.) -dominated mixed forest on VT (*Vaccinium vitis-idaea*) site, later referred as 'VT pine' (Figure 4b).

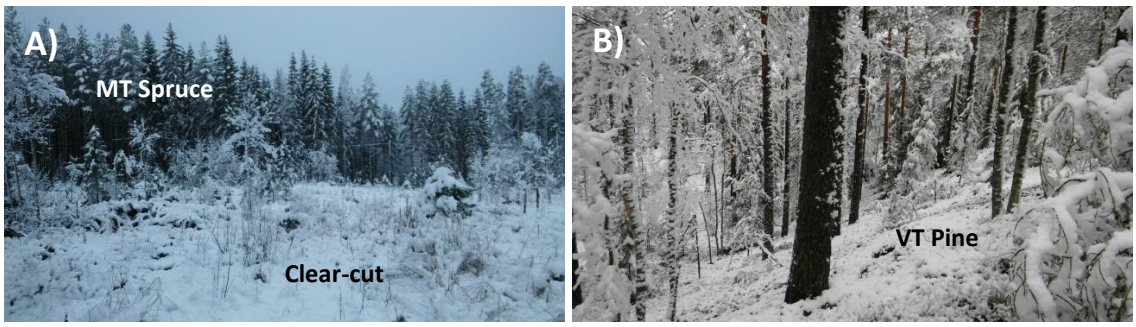

Figure 4. Study sites in Lapinjärvi in November 2017. A) 'Clear-cut' site and the adjacent spruce-dominated site 'MT spruce' in the background. B) Third study site 'VT pine'.

Part of the Lapinjärvi clear-cut site was covered with a logging residue experiment established in September 2014 (Table 1). Experiment has been described and studied by Törmänen *et al.* (2018). Before the final felling, there was a spruce-dominated mixed stand that was approximately 80 years old. Experimental design was randomized block design with fresh logging residue treatments. The treatments were Norway spruce, Scots pine and silver birch logging residues piled on study plots (1.5 m x 2 m = 3 m^2) and corresponding control plots without residues. There were four replicate plots in each treatment. In this study, only Norway spruce plots with 40 kg m^{-2} of logging residues (spruce LR) and control plots (control) without residues were studied.

The Kiikala study site was a nutrient-poor Scots pine stand (Table 1). There was a logging residue experiment established in spring 2007 in connection with thinning on three control plots (30 m x 30 m) of a forest fertilization experiment. These plots had not received any fertilizer input. After the thinning, the logging residues were spread uniformly around randomly selected trees (r = 2.5 m) at four different levels: 0, 2.5, 5.1, and 7.6 kg m^{-2}. Each logging residue level had two replicates at each plot but only one replicate from each plot was investigated in this study, resulting in three replicates per treatment.

Table 1. Descriptions of the logging residue experiment sites in Lapinjärvi and Kiikala.

	Lapinjärvi	Kiikala
Location	Southeastern Finland	Southwestern Finland
Forest management practice	Final felling	Thinning
Year of the management practice	2014	2007
Stand age during the management practice, years	80	53
Tree species	Norway spruce	Scots pine
Site type[a]	MT	CT
Humus type	Mor	Mor
Soil type[b]	Haplic Arenosol	Podzol
Average annual precipitation (mm)[c]	640	680
Annual mean air temperature (°C)[c]	4.6	5.4

Data for the Lapinjärvi site is taken from Törmänen *et al*. (2018); data for Kiikala from Adamczyk *et al*. (2015).
[a]According to Cajander (1949): MT - *Vaccinium myrtillus*; CT - *Calluna vulgaris*. Fertility order of the site types: CT < MT.
[b]According to IUSS Working Group WRB (2006).
[c]Climatic data acquired from FMI (2018); average from 30 years (1981–2010).

6.2 Studying diffusive N fluxes with microdialysis device

6.2.1 Microdialysis system set-up

Two similar microdialysis devices were used for sampling. Each device consisted of three components: syringe pump, microdialysis probe and refrigerated fraction collector (Figure 5). Syringe pump (CMA 4004) was equipped with four microsyringes (2.5 ml). Each syringe was connected to a microdialysis probe (CMA 20 Elite) that has a PAES membrane with 20 kDa MWCO, outer diameter of 500 µm and inner diameter of 400 µm. The flow rate of perfusion fluid inside the probe and cannulae was controlled with the syringe pump. Samples were collected in 300 µl plastic vials with a refrigerated fraction collector (CMA 470) that was adjusted to keep the temperature inside the collector at +6 °C during sampling. All equipment was purchased from CMA Microdialysis AB (Kista, Sweden).

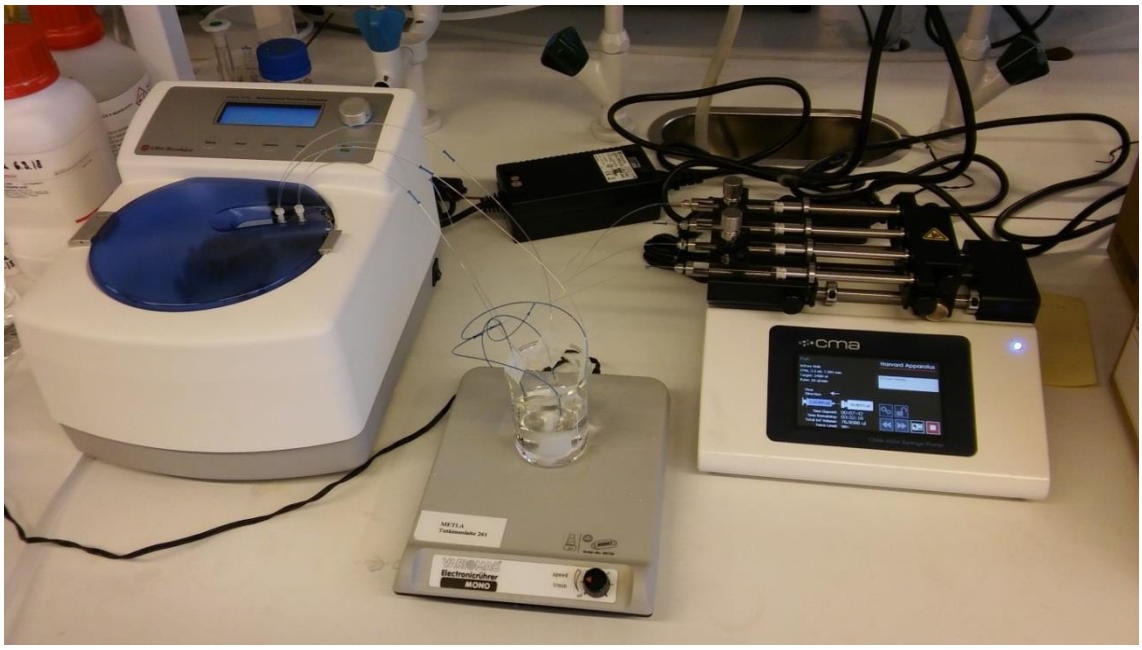

Figure 5. Microdialysis system set-up. From left: refrigerated fraction collector (CMA 470), microdialysis probe (CMA 20) immersed in solution, and syringe pump (CMA 4004).

In *ex situ* microdialysis sampling and experiments, 10 mm long membrane (surface area 0.159 cm^2) was used. Longer membrane (30 mm; surface area 0.4732 cm^2) was chosen for the *in situ* microdialysis sampling as it has been shown to improve the recovery of N compounds from stirred solution as compared to the 10 mm membrane (Buckley et al. 2017).

6.2.2 Calibration of microdialysis probes

It is important to ensure that the relative recovery (RR) of the microdialysis probes for the target compounds stays constant throughout the sampling period to notice any damage or fouling of the membranes (Inselsbacher et al. 2011). Prior to the soil sampling, microdialysis probes were calibrated following the earlier established microdialysis probe calibration procedure (Bungay et al. 1990, Inselsbacher et al. 2011). Probes with 10 mm membrane were immersed in N standard solution containing NH_4-N as ammonium chloride (NH_4Cl, Merck, Darmstadt, Germany), NO_3-N as potassium nitrate (KNO_3, Merck) and amino acid-N as glycine (Gly, Sigma-Aldrich Co., St. Louis, MO, USA) and L-glutamic acid (Glu, Sigma-Aldrich) 100 µmol l^{-1} each. Solution

contained also additional amino acid-N as L-glutamine (Gln, Sigma-Aldrich) and L-lysine (Lys, Sigma-Aldrich) 400 µmol l^{-1} each. Concentrations of the N compounds and the amino acids under scrutiny were chosen based on the calibration procedures of the earlier studies (e.g. Inselsbacher et al. 2011, Buckley et al. 2017). Probes were perfused with Milli-Q water at flow rates of 1, 3, 5, 7 and 10 µl min^{-1} over a sampling period of 260 to 26 minutes, the time depending on the flow rate so that the resulting dialysate volume was 260 µl. Solution was stirred with a magnetic stirrer (Variomag®, Daytona Beach, FL, USA) throughout the calibration period. Dialysates were frozen (-18 °C) until the analyses. The relative recovery was calculated (Equation 2) as described by Inselsbacher *et al*. (2011):

$$RR = 100 \times \frac{C_{dial}}{C_{std}}, \quad (2)$$

where

RR = relative recovery (%)

C_{dial} = concentration of target N compound measured from the dialysate (mol l^{-1})

C_{std} = concentration of the compound in the N standard solution (mol l^{-1}).

The first calibration was unsuccessful for the calculation of the relative recoveries of NH_4^+ and the total free amino acids due to the high concentration of amino acids in the solution that caused unmeasurable values in amino acid analysis as well as potential error in ammonium analysis. Therefore, additional microdialysis probe calibrations were run with two different solutions containing either NH_4-N 100 µmol l^{-1} or amino acid-N as Gly, Glu, Gln and Lys 100 µmol l^{-1} each. Solutions were sampled at flow rates of 1, 5, and 10 µl min^{-1}, resulting in dialysate volumes of 120 µl and 100 µl for the determination of the RR of NH_4^+ and amino acids, respectively. Solutions were stirred with a magnetic stirrer and covered with a parafilm throughout the sampling period. Samples (200 µl) were taken from both solutions before and after each sampling period to measure the initial and final concentration of NH_4^+ and amino acids.

The diffusive flux of the target compound across the membrane surface over time was calculated (Equation 3) according to Leitner *et al*. (2017):

$$F_{MD} = \frac{C_{dial} \times V}{A_{MD} \times t}, \quad (3)$$

where

F_{MD} = diffusive flux (mol cm^{-2} h^{-1})

C_{dial} = concentration of target N compound measured from the dialysate (mol l^{-1})

V = sample volume (l)

A_{MD} = membrane surface area (cm^2)

t = sampling time (h).

For *ex situ* experiments and *in situ* soil sampling, flow rate 5.0 µl min^{-1} was chosen as it was shown to result in an adequate concentration of target compounds in the dialysate within a reasonable sampling time (52 min). Prior to each sampling event, it is important to collect a small amount of sample into a waste vial for approximately 10–20 minutes to ensure that the dead volume has been removed from the cannulae. Blank samples were prepared for each measurement by placing the microdialysis probes in Milli-Q water that was stirred with a magnetic stirrer and perfusing the probes at a flow rate of 5 µl min^{-1}. The RR of 30 mm membranes for target N compounds was verified before and after each *in situ* soil sampling event by immersing the membranes in a stirred N standard solution containing 50 µmol N l^{-1} of each studied compound (NH_4^+, NO_3^-, Gly, Gln, Glu and Lys) and perfusing them with Milli-Q water at a flow rate 5 µl min^{-1}. Dialysates were stored at -18 °C until the analyses of ammonium, nitrate and the total free amino acids (Section 6.2.5).

6.2.3 *Ex situ* soil sampling with microdialysis device

For the *ex situ* microdialysis experiments, organic (O) horizon of the chosen study sites (clear-cut, MT spruce and VT pine in Lapinjärvi) was sampled with a soil core (d = 58 mm) in November 2017. Five soil cores were sampled and combined to give a composite sample for each site. Samples were stored at +4 °C for sieving and the analyses of the basic soil properties as described below (Section 6.4.2). For the *ex situ*

microdialysis, sieved soil samples were stored at -18 °C. *Ex situ* microdialysis experiments were run at room temperature for the sieved (ø 6 mm) soil samples.

Ex situ microdialysis sampling was run by placing the four microdialysis probes (10 mm membrane) vertically into the soil at 1.5 cm depth from the soil surface and perfusing them with Milli-Q water at a flow rate of 5 µl min^{-1} for 52 min. To get a volume of 100 ml, following amounts of different soil samples were weighed based on their 'moist bulk density' (determined by weighing 20 ml of fresh and sieved soil) into glass beakers (100 ml): 54 g (clear-cut), 43 g (MT spruce) and 36 g (VT pine). To install the microdialysis probes, the soil had to be slightly compressed. Diffusive N fluxes were determined from two replicate beakers resulting in eight replicate dialysate samples for each soil sample.

The effect of soil moisture content on N fluxes was tested by adding either 10 g or 35 g of Milli-Q water at the surface of the 'MT spruce' soil sample (43 g in a 100 ml glass beaker) to achieve a moisture content of 60 % of water-holding capacity (WHC) or 100 % WHC, respectively. The soil sample had been stored at +4 °C in plastic bag for one month and had a moisture content of approximately 44 % WHC. It is possible that the soil moisture content had slightly changed during the storage but it was not checked. After the water additions, the three soil microcosms (44 % WHC, 60 % WHC and 100 % WHC) were covered with parafilm and stored at +4 °C for a few hours before the microdialysis sampling to get the soil samples moistened. There was only one replicate beaker at each moisture level but otherwise the microdialysis sampling was run as described above.

Small-scale temporal changes in nitrogen fluxes were measured in a nitrogen addition experiment that was run for all three soil samples by weighing following amounts of the soil samples into glass beakers (100 ml): 54 g (Clear-cut), 43 g (MT spruce) and 36 g (VT pine). Following amounts of Milli-Q water were added to the soil samples on the day before the N addition experiment: 0.8 g (Clear-cut), 6.0 g (MT spruce) and 9.2 g (VT pine) to reach moisture content of 60 % WHC after the addition of N solution. Four milliliters of nitrogen standard solution containing 50 µmol N l^{-1} of NH_4^+, NO_3^-, Gly, Gln, Glu and Lys was added to the soil surface after 52 min microdialysis sampling that

was run to measure the initial N fluxes in soil samples. Three subsequent dialysate samples were collected after N addition in 52 min intervals during a total sampling period of 2 h 36 min. Dialysates were stored at -18 °C until the analyses of ammonium, nitrate and the total free amino acids (Section 6.2.5).

6.2.4 *In situ* soil sampling with microdialysis device

In situ microdialysis and soil sampling for further analyses were done in Lapinjärvi in June 2018 and in Kiikala in September 2018. At the Lapinjärvi logging residue experiment, samples were collected from a corner of each plot chosen to this study: Norway spruce (40 kg m^{-2}) and control treatments with four replicate plots each. Also, four replicate samples were taken from the 'MT spruce' site; two of the sampling plots here happened to be dominated by feathermoss (*Pleurozium schreberi*), and the other two were dominated by shrubs, mostly bilberry (*Vaccinium myrtillus* L.). At the Kiikala logging residue experiment, sampling spot was chosen randomly at one-meter radius from each tree studied; there were three replicates for each logging residue level (0, 2.5, 5.1, 7.6 kg pine logging residues m^{-2} around each tree).

Sampling procedure was similar at both study sites (Lapinjärvi and Kiikala) and at all spots and it was done in the following order: (1) sampling of the diffusive fluxes of NH_4^+, NO_3^- and amino acids with the microdialysis device (Figure 6a), (2) collection of volatile monoterpenes from the same spot (described below in Section 6.3; Figure 6b) and (3) taking the soil core to the lab for further analyses (described below in Section 6.4; Figure 6c).

Figure 6. Sampling at the Lapinjärvi study site in June 2018. A) *In situ* microdialysis sampling at the logging residue experiment (Norway spruce 40 kg m^{-2} treatment). B) Collecting volatile monoterpenes from the soil air. C) Soil core that was taken to the lab for further analyses.

Before the *in situ* microdialysis sampling, above-ground vegetation and coarse litter were carefully removed and four microdialysis probes (with 30 mm membrane) were vertically installed into the organic horizon (O_{fh}; fermentation (f) and humus (h) layers were under scrutiny) at 1.5 cm depth (Figure 7). Flow rate was 5 µl min^{-1}, sampling time 52 min and the temperature in the fraction collector was adjusted to +6 °C. Four replicate microdialysis samples were collected from each spot studied (n = 16 for each treatment in Lapinjärvi and n = 12 for each logging residue level in Kiikala). Dialysates were stored at -18 °C until the analyses of ammonium, nitrate and the total free amino acids (Section 6.2.5).

Figure 7. Microdialysis probes in soil.

6.2.5 Determination of $NH4^+$, NO_3^- and total free amino acids from dialysates

Ammonium was quantified from the dialysate samples using a modified indophenol method as described by Hood-Nowotny *et al*. (2010) with a few modifications. Modified indophenol method is based on a Berthelot reaction where NH_4^+ is oxidized by sodium hypochlorite to monochloroamine that reacts with phenolics in high pH and forms a green indophenol compound (Kandeler & Gerber 1988). A color reagent was prepared weekly by dissolving 6.8 g of sodium salicylate (Merck) and 0.025 g of sodium nitroprusside (Merck) in 100 ml of Milli-Q water. An oxidation reagent was prepared daily from 0.2 ml of sodium hypochlorite (14 % Cl_2 in aqueous solution, VWR, Fontenay-sois-Bois, France) and 9.8 ml of 1.5 M sodium hydroxide (NaOH, Sigma-Aldrich). Standards 2–200 µmol N l^{-1} were prepared on the measurement day from NH_4Cl stock solution. For the measurements, 80 µl of each standard as two replicates, blanks, and samples were pipetted into a flat-bottomed, transparent 96-well microplate (SpectraPlate, PerkinElmer Inc.), followed by addition of 60 µl of color reagent and 60 µl of oxidation reagent. Plate was incubated at room temperature shielded from light for 50 min. Absorbance was measured at wavelength of 655 nm with a microplate reader Infinite M200 (Tecan Group Ltd, Männedorf, Switzerland)

from the dialysates of the microdialysis probe calibration (Section 7.1) and from the dialysates of the *ex situ* microdialysis sampling for the initial diffusive fluxes (Section 7.2.2) or with CLARIOstar (BMG Labtech, Ortenberg, Germany) from the rest of the dialysates.

Nitrate concentrations in the dialysates were determined by Vanadium-Griess method (Miranda et al. 2001, Hood-Nowotny et al. 2010), with modifications by Inselsbacher *et al*. (2011). Nitrate is reduced to nitrite in an acidic vanadium (III) chloride (VCl_3) solution and nitrite concentration is measured after linking to the Griess reaction. Griess reagent 1 was prepared by dissolving N-(1-naphthyl) ethylenediamine dihydrochloride (Thermo Fisher, Karlsruhe, Germany) into Milli-Q water. The Griess reagent 2 was prepared by dissolving sulfanilamide (Merck) into 3 M hydrochloric acid (HCl 37 %, J.T. Baker, Deventer, the Netherlands). A saturated VCl_3 (Merck, France) reagent was prepared weekly by dissolving 400 mg VCl_3 into 50 ml 1 M HCl. Depart from the followed procedure, the VCl_3 reagent was not filtered since there were no visible solids left after a proper mixing. Standards 1–100 µmol N l^{-1} were prepared daily from KNO_3 stock solution. Griess reagents were mixed in equal amounts just before being added to the assay mixtures. For the analysis, 50 µl of samples, blanks, and two replicates of each standard were pipetted into a transparent 96-well microplate, followed by 50 µl of VCl_3 reagent and 50 µl of the mixture of the Griess reagents. The plate was then incubated at 37 °C in dark for 60 min before the absorbance measurement with microplate reader (same devices as described above for the ammonium determination) at 540 nm.

The total free amino acids were analyzed from the dialysates as described by Jones *et al*. (2002), with the modifications for the use in microplate analysis by Darrouzet-Nardi *et al*. (2013). Briefly, *o*-phthaldialdehyde (OPA) and *β*-mercaptoethanol (ME) working reagent was prepared by adding OPA (Thermo Fisher, Rockford, USA) to methanol (HPLC-grade, Merck, Germany), followed by 0.02 M potassium tetraborate (Honeywell, Seelze, Germany) that was adjusted to pH 9.5 with 10 M potassium hydroxide (Merck, Germany). Finally, ME (VWR, Solon, OH, USA) was added to this OPA-1, and the resulting solution was called OPAME reagent. The reagent was left in room temperature shielded from light for at least two hours to minimize the effect of background fluorescence. Standards 5–500 µmol N l^{-1} were prepared daily from glycine

stock solution. For the analysis, 50 µl of samples, blanks, and two replicates of each standard were pipetted into a black 96-well microplate, followed by 100 µl of OPAME reagent. The plate was then incubated at room temperature in dark for 60 min before the fluorescence measurement with microplate reader (same devices as described above for the ammonium determination). Excitation wavelength was set to 360 nm, emission wavelength 460 nm, and sensitivity was 50.

6.3 Studying volatile monoterpenes in soil atmosphere

After the *in situ* microdialysis sampling (Section 6.2.4), volatile monoterpenes were collected from the soil air at the same sampling spot on which *in situ* microdialysis sampling was done, using a chamber method (Haselmann et al. 2000) as described by Smolander *et al*. (2006). This resulted in one replicate volatile monoterpene sample from each spot studied (n = 4 for each treatment in Lapinjärvi and n = 3 for each logging residue level in Kiikala). Briefly, a stainless-steel cylindrical cap (diameter = 19 cm, height = 12 cm) was hammered as deep as possible into soil. A sorbent sampling tube (active carbon, Anasorb CSC, SKC, 84, PA, USA) was connected to the chamber and soil air was sampled by pumping it through the tube at a flow rate of 0.5 l min^{-1} for 6 min. Temperature was measured from the middle of the organic horizon at a spot close to the chamber. Sampling tubes were stored at -18 °C until the analyses.

Monoterpenes were later extracted from the active carbon tubes with dichloromethane (VWR, France), followed by the measurement in the laboratory by gas chromatography - mass spectrometry (GC-MS) with a system consisting of 7693 Autosampler, 7890B Gas Chromatograph equipped with a DB-5MS capillary column (length 30 m x ID 0.25 mm x film 0.25 µm) and 5977A Mass Spectrometer (Agilent technologies, Santa Clara, CA, USA). Monoterpenes were analyzed directly from the dichloromethane solution with GC-MS, using following conditions: initial temperature 30 °C, rate 10 °C min^{-1}, hold time 5 min; rate 40 °C min^{-1}, hold time 2 min; final temperature 230 °C. Temperature at MS interface was 230 °C (ion source: 230 °C). Compound identification was based on Agilent Chemstation software with mass spectral libraries. Quantitative analyses were performed based on internal standard 1-chlorodecane (Fluka, Switzerland) and external standard humulene (Fluka).

6.4 Soil sampling, pretreatment and analyses

Soil samples taken from Lapinjärvi in November 2017 for the *ex situ* microdialysis sampling were analyzed only for the basic soil characteristics (soil moisture content, organic matter content, WHC, pH and C-to-N ratio) as described below. Soil samples taken from Lapinjärvi in June 2018 and from Kiikala in September 2018 in connection with the *in situ* microdialysis sampling (Section 6.2.4) were analyzed for the basic soil characteristics, KCl-extractable NH_4-N and NO_3-N, N transformations, microbial biomass C and N and condensed tannins. A summary of the study sites, treatments and analyses is given in Appendix 1.

6.4.1 Soil sampling

Soil core from the cylindrical cap resulting from the collection of volatile monoterpenes in soil air as described above (Section 6.3) was taken to the lab for further analyses. This resulted in one replicate soil sample from each spot studied (n = 4 for each treatment in Lapinjärvi and n = 3 for each treatment in Kiikala). Samples were stored at +4 °C until the analyses.

6.4.2 Pretreatment of soil and determination of basic characteristics

Litter layer and mineral soil were carefully removed from all of the soil cores at the laboratory during the following 1–2 d after soil sampling. Organic horizon (O_{fh}) samples were sieved (ø 6 mm) and mixed for the analyses. Basic soil properties were determined for all the soil samples. First, 3 g of fresh soil was dried overnight at 105 °C to calculate soil moisture content (m-%) by dividing the mass of water (m_w) vaporized from the sample by the mass of sample after drying that is assumed to be the mass of soil solids (m_s). The same sample was then incinerated at 550 °C for 4 h to determine the organic matter content as loss-on-ignition. Water-holding capacity was determined for 20 ml of fresh soil by soaking it with 50 ml of Milli-Q water in a funnel with black ribbon filter paper (S&S, 589/1, diameter 185 mm) for 2 h. The amount of water filtered was subtracted from the amount of water added in soil. The resulting water amount was

assumed to be adsorbed by soil. It was added to the initial water content, and the result was assumed to be 100 % WHC. Soil pH was measured from a soil-water suspension (15 ml/25 ml) using pH meter (pH 1000 L, VWR, Leuven, Germany). Total C and N were measured from air-dried and ground (d = 0.5 mm) soil using an automated CHN analyzer (CHN-600, Leco, Saint Joseph, MI, USA).

6.4.3 Determination of soil KCl-extractable NH_4^+-N and NO_3^--N and N transformations

Nitrogen transformations were measured from the soil samples in an incubation experiment described by Smolander *et al*. (1995) with the modifications by Törmänen *et al*. (2018). Shortly, an amount that represents fresh and sieved soil volume of 20 ml was weighed into a 125 ml infusion bottle and soil moisture was adjusted to 60 % WHC. Two replicates of each soil sample were stored in -18 °C until KCl extraction. Other two replicates of each sample were kept in a constant temperature (+14 °C) and moisture content (60 % WHC) covered with aluminum foil for 42 d. These samples were frozen as well after the incubation period to ensure the comparability to the results obtained from non-incubated samples. After this, each incubated and non-incubated sample was shaken with 40 ml of 1 M KCl (Merck, Germany) for 2 h (200 rpm) with a table shaker (Certomat R, B. Braun, Melsungen, Germany) and filtered through a blue-ribbon filter paper (S&S, 589/3). Concentrations of NH_4^+-N and NO_3^--N in the KCl-extracts were measured with a flow injector analyzer (QuickChem FIA+ 8000 Series, Lachat).

Determination of NH_4^+-N by flow injection technique is based on injection of the sample into the carrier (deionized water) flow stream after which it is mixed with NaOH. Mixture flows along with the polytetrafluoroethylene (PTFE) membrane in gas diffusion block, ammonia gas is formed and diffused across the membrane into the indicator flow stream that changes its color, and finally intensity is measured at wavelength 590 nm spectrophotometrically. In the determination of NO_3^--N, in turn, sample is injected in water and mixed with NH_4Cl buffer. By using flow injection technique, NO_3^- is reduced to NO_2^- in cadmium column, after which it reacts with the sulfanilamide color reagent that contains also N-(1-naphthyl) ethylenediamine dihydrochloride. The intensity of the formed azo compound is measured

spectrophotometrically at wavelength 520 nm. Net N mineralization rate was calculated from the results by subtracting the total concentration of mineral N (NH_4^+-N and NO_3^--N) in the non-incubated samples from that in the incubated samples and dividing it by the incubation time (days). Result was then calculated on soil organic matter (o.m.) basis and expressed as mg N kg^{-1} o.m. d^{-1}. Net nitrification rate was calculated in a similar way from the NO_3^--N results.

6.4.4 Determination of microbial biomass C and N by fumigation-extraction

The concentrations of C and N in the soil microbial biomass were examined by fumigation-extraction as described by Martikainen and Palojärvi (1990), with modifications by Törmänen *et al*. (2018). Briefly, an amount that represents fresh and sieved soil volume of 20 ml was weighed into a 125 ml infusion bottle and soil samples were moistened to 60 % WHC. Two replicate samples were kept at +4 °C as controls that were measured for the dissolved organic carbon (DOC) and the total dissolved nitrogen (TDN) contents and the other two replicates were fumigated with chloroform (Merck, Germany) in open infusion bottles in desiccator for 24 h at 28 °C. All samples were extracted with 50 ml of 0.5 M potassium sulfate (K_2SO_4, Merck) for 30 min at 200 rpm on a table shaker. Extracts were filtered through blue ribbon filter paper (S&S, 589/3) and kept at -18 °C until the measurements of C and N with a total organic carbon analyzer (TOC-$V_{CPH/N}$ analyzer, Shimadzu, Japan). For the blank samples, 50 ml of 0.5 M K_2SO_4 was filtered. The differences between the concentrations of C (FE-C, µg g^{-1} d.m.) and N (FE-N, µg g^{-1} d.m.) in fumigated and unfumigated samples were converted to microbial biomass C (C_{mic}; Equation 4) and N (N_{mic}; Equation 5) by using the following formulae (Martikainen & Palojärvi 1990):

$$C_{mic} = (1{,}9 \times FE\text{-}C + 428)\mu g\ g^{-1}\ d.m. \text{ and} \quad (4)$$

$$N_{mic} = (1{,}86 \times FE\text{-}N + 74{,}82)\mu g\ g^{-1}\ d.m. \quad (5).$$

6.4.5 Determination of condensed tannins by acid-butanol assay

Condensed tannins were determined from air-dried, ground (ø 0.5 mm) soil samples. For the extraction, 0.50 g of each soil sample as three replicates was weighed into a tube and extracted three times with 20 ml of 70 % aqueous acetone (70 % acetone, Merck, Germany and 30 % water) for 10 min with the accelerated solvent extractor (Dionex ASE 350, Thermo Fisher, Sunnyvale, CA, USA). Temperature was 40 °C and pressure raised up to 100 bars during the extraction. Extracts were transferred into beakers and the liquid was let to evaporate in a fume cupboard. Finally, condensed tannins were determined by acid-butanol assay (Porter et al. 1986, Terrill et al. 1992) as described by Smolander *et al*. (2005a). Acid-butanol assay is based on the oxidation of proanthocyanidins (condensed tannins) in *n*-BuOH (butan-1-ol, VWR) - HCl (95:5) solution, followed by colorimetric determination. Previous standard curves that had been prepared from extracted and purified condensed tannins from silver birch leaves and Norway spruce needles as described by for example, Adamczyk *et al*. (2011) were used. Condensed tannins were measured from the samples with a spectrophotometer (UV-2401 Pc, UV-VIS Recording Spectrophotometer, Shimadzu, Japan) at the wavelength of 555 nm.

6.5 Statistical analyses

Normality of the analyzed data was tested with Shapiro-Wilk and Kolmogorov-Smirnov test with Lilliefors significance correction. In the cases where the variables were not normally distributed, the non-parametric Kruskal-Wallis test was used. Statistically significant differences were determined using p-value of 0.05. For the *ex situ* microdialysis results, Kruskal-Wallis was used to determine whether there were significant differences in the soil properties or the diffusive fluxes (initial and at different moisture levels) of different N forms across samples taken from Lapinjärvi in 2017. For the data from the *in situ* microdialysis and sampling in Lapinjärvi in 2018, Kruskal-Wallis test was used to determine if there were any significant differences between the treatments. Since soil organic matter content in the samples taken from Lapinjärvi in 2018 followed normal distribution and the Levene's test showed that the

homogeneity of variances was satisfied, one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) as a Post-Hoc test was performed.

To determine whether there was any linear relationship between the amount of pine logging residue in the Kiikala experiment and the variables studied, Pearson's correlation coefficient was used. Pearson's correlation coefficient and the p-value of the 2-tailed test were used to study also, if there were any statistically significant linear relationships between the studied variables in the data from Lapinjärvi and Kiikala in 2018. Statistical analyses were done with SPSS software (version 25.0, IBM Corp., New York, USA).

7 RESULTS

7.1 Relative recoveries of ammonium, nitrate and the total free amino acids

The relative recovery of ammonium and nitrate from the N standard solution sampled with 10 mm long microdialysis membrane decreased exponentially with increasing flow rate (Figure 8; equations for regression curves are given in Table 2). Respectively, the absolute recovery that is the flux (nmol N cm^{-2} h^{-1}) of target compounds from the standard solution, increased with increasing flow rate. Similar pattern was detected for the total free amino acids as well (data not shown). Flow rate of 5 µl min^{-1} was chosen for further experiments since it was shown to be adequate in terms of gaining detectable concentrations of target compounds in a reasonable sampling time.

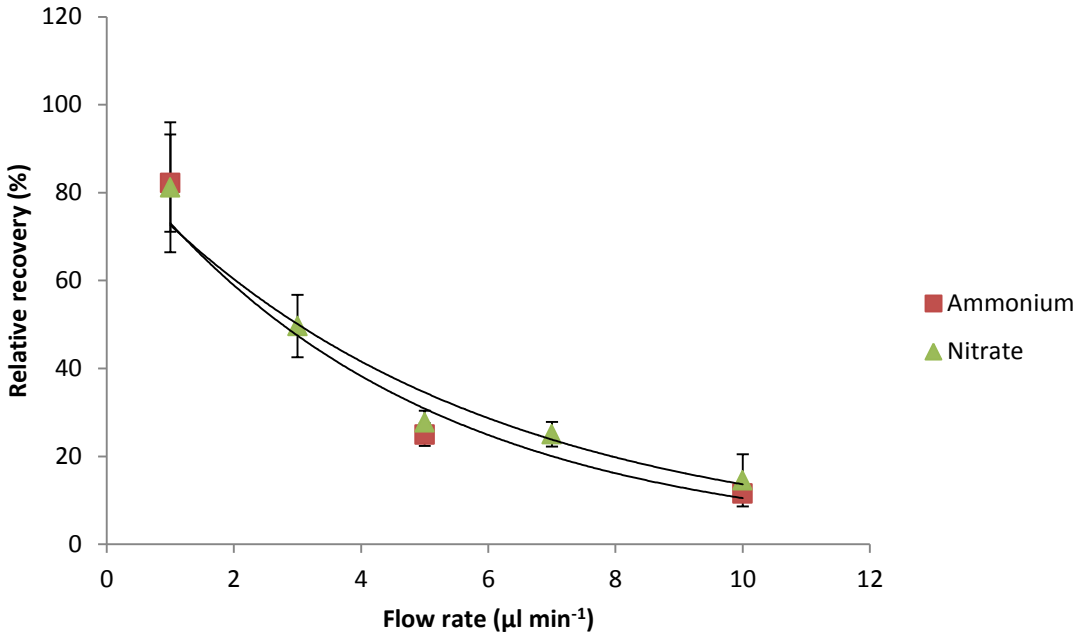

Figure 8. The effect of flow rate on relative recovery of ammonium and nitrate was studied by immersing 10 mm microdialysis membranes in stirred N solutions containing different amounts of target compounds; nitrate recovery was studied from a solution containing 100 µmol N l^{-1} of NH_4^+ and NO_3^- as well as 1000 µmol N l^{-1} of free amino acids. Separate solutions containing 100 µmol N l^{-1} of NH_4^+ were used to study the relative recovery of NH_4^+. Results are expressed as averages over replicates ± SD (n = 4). Equations for regression curves are given in Table 2.

Table 2. Equations of regression curves for the relative recoveries (RR) for NH_4^+ and NO_3^- as a function of flow rate (FR). Data is given in Figure 8.

	Relative Recovery	R^2
NH_4^+	$RR = 90.636 \times \exp(-0.215 \times FR)$	0.97
NO_3^-	$RR = 87.465 \times \exp(-0.186 \times FR)$	0.96

The relative recoveries of different N compounds with 30 mm microdialysis membrane sampled from a stirred N standard solution containing 50 µmol N l^{-1} of each studied compound (NH_4^+, NO_3^-, Gly, Gln, Glu, Lys) at a flow rate 5 µl min^{-1} stayed relatively constant over the sampling events (data not shown). On average, the relative recoveries were 73 ± 6 % (n = 16) for ammonium, 76 ± 12 % (n = 16) for nitrate and 64 ± 8 % (n = 8) for amino acids, being substantially higher than the RRs gained at a same flow rate with shorter, 10 mm membranes where they were less than 30 % for NH_4^+ and NO_3^-.

Results from the additional calibration (described in section 6.2.2) showed that amino acid method measured approximately 8.2 ± 0.7 % of the NH_4^+ from a 100 µmol N l^{-1} NH_4^+-solution. However, even though ammonium may have affected the microdialysis probe calibration results for the relative recoveries of amino acids, it was not probably a problem in the *in situ* microdialysis samples where the ammonium concentrations were relatively small. It was also detected that the ammonium concentrations increased slightly (4.7 ± 3.2 %) in the solution during the sampling period but this seemed not to be connected to the sampling time as the highest increase was detected with 10 µl min^{-1} flow rate when the sampling time was the shortest (data not shown).

7.2 *Ex situ* microdialysis

7.2.1 Basic soil characteristics

The soil samples taken from Lapinjärvi in 2017 for the *ex situ* microdialysis did not show any statistically significant differences between the different sites in pH value, gravimetric moisture content, organic matter content or C-to-N ratio (Table 3; $p > 0.05$). Even though not statistically significantly probably due to the small amount of data, VT pine site had approximately 58 % higher soil moisture content and 43 % higher organic matter content in the O_{fh} horizon than the other two sites on average.

Table 3. Soil pH, moisture content, organic matter content and C-to-N ratio in the O_{fh} horizon of different sites in Lapinjärvi in November 2017. Results are expressed as averages over replicates ± SD (n = 2). For each variable, the values marked with the same letter do not differ statistically from each other (Kruskal-Wallis, $p < 0.05$). Data for C-to-N ratio could not be statistically analyzed, as there was only one replicate value for each site.

Study site	pH (H_2O)	Moisture (m-%)	Organic matter (%)	C-to-N ratio
Clear-cut	4.2 ± 0.02[a]	190 ± 6[a]	48 ± 7[a]	31
MT spruce	3.9 ± 0.01[a]	190 ± 7[a]	53 ± 3[a]	32
VT pine	4.1 ± 0.01[a]	300 ± 17[a]	72 ± 2[a]	33

7.2.2 Diffusive N fluxes

There were no statistically significant differences across the diffusive N fluxes of different sites (Figure 9). However, the total amino acid (AA_{TOT}) flux was almost 2-fold in MT spruce site, as compared to the adjacent clear-cut (Figure 9a). Ammonium flux was highest at the VT pine site (Figure 9b), although not statistically significantly. The clear-cut site had more than two times higher nitrate flux than the MT spruce site (Figure 9c) but the difference was not statistically significant because of the large variation. Total free amino acids contributed most (58–75 %) to the total N flux at all sites and nitrate contributed less than 2 % in clear-cut or even 0 % in VT pine.

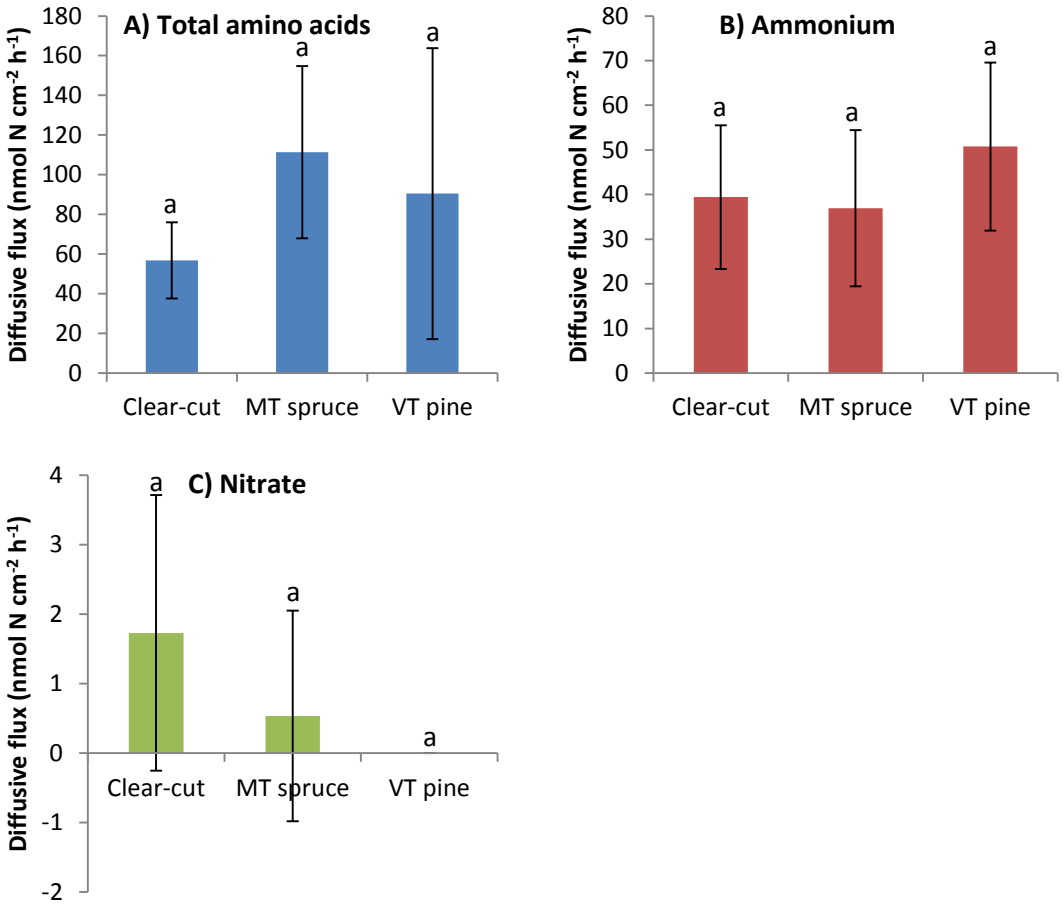

Figure 9. Fluxes of A) total free amino acids, B) ammonium and C) nitrate at the different sites in Lapinjärvi in November 2017 sampled *ex situ* from sieved organic horizon (O_{fh}) soil with microdialysis device. Results are expressed as averages over replicates ± SD (n = 8). For each N form, the values marked with the same letter do not differ statistically from each other (Kruskal-Wallis, $p < 0.05$).

7.2.3 Effect of soil moisture content on diffusive N fluxes

The diffusive fluxes of the total free amino acids in the 'MT spruce' sample taken from Lapinjärvi in 2017 did not differ from each other at the studied soil moisture content levels (Figure 10a). The average ammonium fluxes were higher in moistened soils than in the field-moist soil (44 % WHC) but the difference was significant at 60 % WHC only (Figure 10b; $p < 0.05$). The diffusive fluxes of nitrate did not show any significant differences between the moisture contents since the variation was very large (Figure 10c).

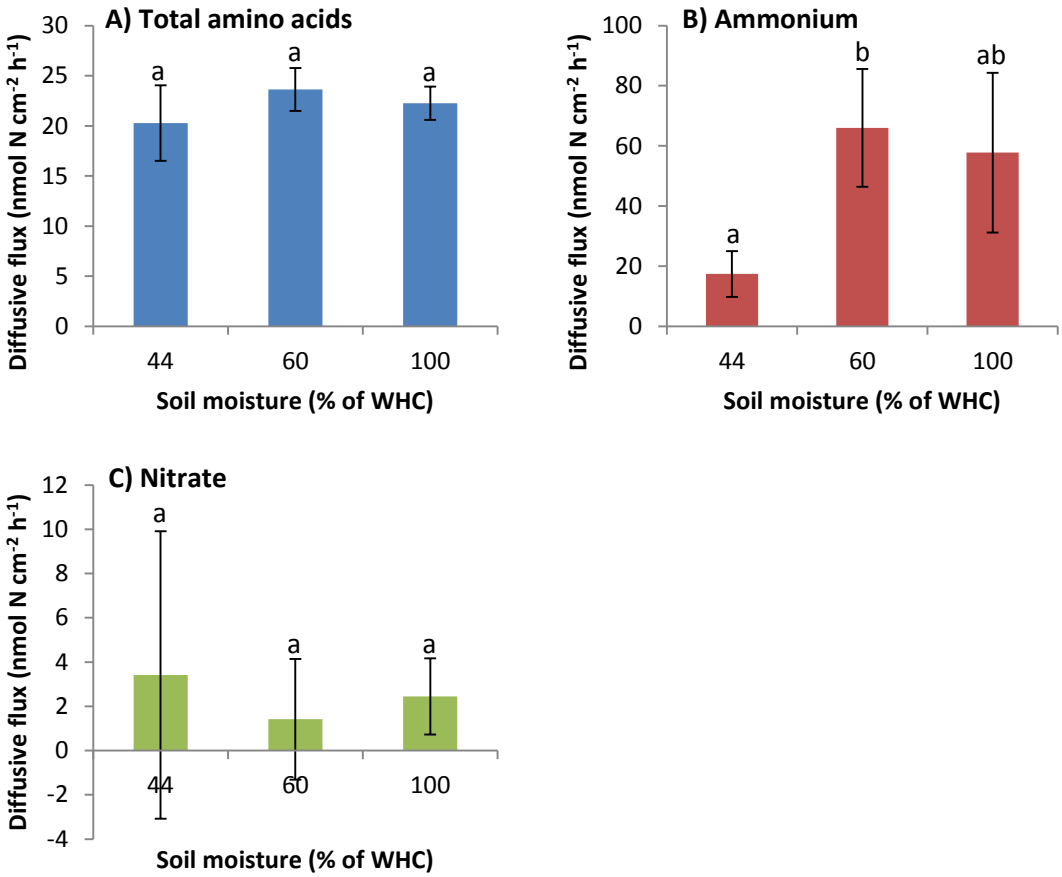

Figure 10. The diffusive fluxes of A) total free amino acids, B) ammonium and C) nitrate at different soil moisture content levels expressed as a percentage (%) of water-holding capacity (WHC) in a sieved organic horizon (O_{fh}) soil taken from Lapinjärvi MT spruce site in November 2017, sampled *ex situ* with microdialysis device. Results are expressed as averages over replicates ± SD (n = 4). For each N species, statistically significant differences between the soil moisture contents are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

7.2.4 Temporal changes in diffusive N fluxes

Temporal changes in the diffusive N fluxes were monitored with the microdialysis device for 156 min after the addition of 4 ml of N solution (50 µmol N l^{-1} of NH_4^+, NO_3^-, Gly, Glu, Gln and Lys) in the sieved soils sampled from the different sites in Lapinjärvi in 2017. The diffusive fluxes of ammonium showed an expected pulse that was followed by a subsequent decrease in all of the studied soil samples (Figure 11). The diffusive flux of the total free amino acids increased remarkably only in the 'VT pine' soil where the flux was 26 ± 21 % higher after 52 min of microdialysis sampling compared to the initial flux (data not shown). Increase in the diffusive nitrate flux was observed only in the clear-cut sample, where the flux increased from 0.9 ± 1.1 nmol N cm^{-1} h^{-1} (0 min from N addition) to 4.1 ± 0.5 nmol N cm^{-1} h^{-1} (52 min from N addition), followed by a subsequent decrease to the initial level. In MT spruce sample, the initial total N flux was as high as after N addition.

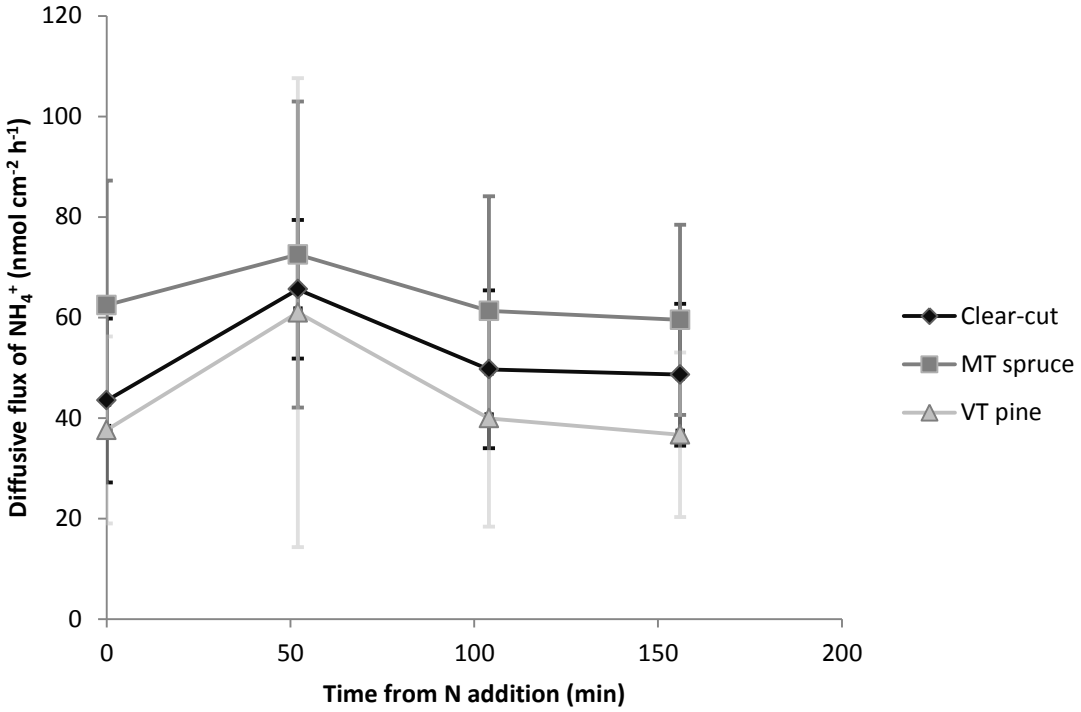

Figure 11. Temporal changes in ammonium fluxes after the addition of nitrogen solution (50 µmol N l^{-1} of NH_4^+, NO_3^-, Gly, Glu, Gln and Lys) in soil samples from different sites in Lapinjärvi (Clear-cut, MT spruce and VT pine). Results are expressed as averages over replicates ± SD (n = 4).

7.3 Diffusive N fluxes sampled *in situ* boreal forest soil

In situ soil sampling with the microdialysis device at the Lapinjärvi study site did not show any statistically significant differences in the diffusive N fluxes of different treatments (Figure 12). However, the control plots of the logging residue experiment had substantially lower mean flux of the total free amino acids than the soil underneath the spruce logging residue piles or at the MT spruce site (Figure 12a). Ammonium fluxes were higher under the logging residue piles than at the control plots or in the forest (Figure 12b), although not statistically significantly. Nitrate fluxes were relatively similar at all treatments (Figure 12c). The share of amino acids of the total N flux was highest at all treatments. At the MT spruce site, the moss-dominated two study plots had substantially higher diffusive AA_{TOT} and NH_4^+ fluxes whereas soil NO_3^- flux was lower, as compared to the two study plots with shrub-dominated ground vegetation (Appendix 2).

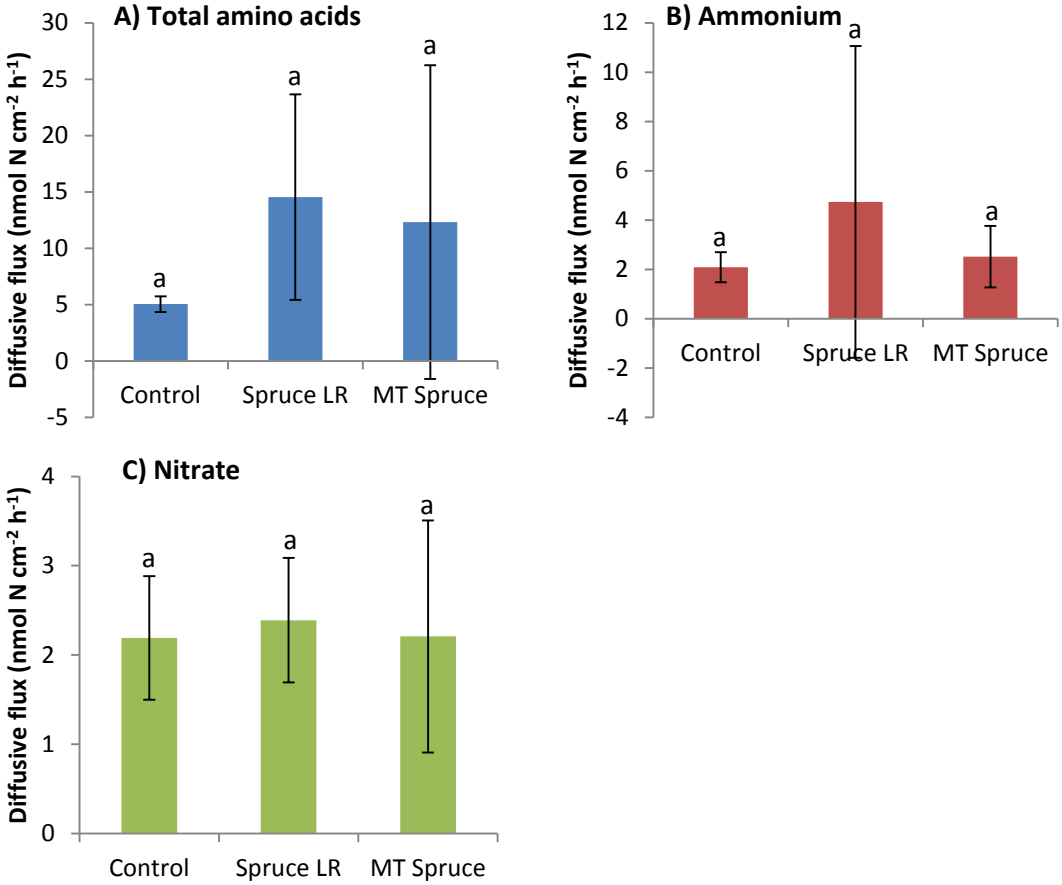

Figure 12. Soil A) total free amino acid, B) ammonium and C) nitrate fluxes in O_{fh} horizon sampled *in situ* with the microdialysis device at different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue. Results are expressed as averages over replicates ± SD (n = 4 for total amino acids, n = 15–16 for NH_4^+ and NO_3^-). For each N form, the values marked with the same letter do not differ statistically from each other (Kruskal-Wallis, $p < 0.05$).

At the Kiikala logging residue experiment, the amount of pine logging residue did not affect statistically significantly soil N fluxes (Figure 13). However, the total free amino acids contributed most to the total diffusive N fluxes at all logging residue levels whereas nitrate flux was small. Pearson correlation coefficients between the amount of pine logging residue and the diffusive N fluxes were as follows: 0.125 for the total free amino acids (Figure 13a; n = 41; p-value of 2-tailed test = 0.436), 0.155 for ammonium (Figure 13b; n = 42; p-value of 2-tailed test = 0.328) and -0.282 for nitrate (Figure 13c; n = 44; p-value of 2-tailed test = 0.063).

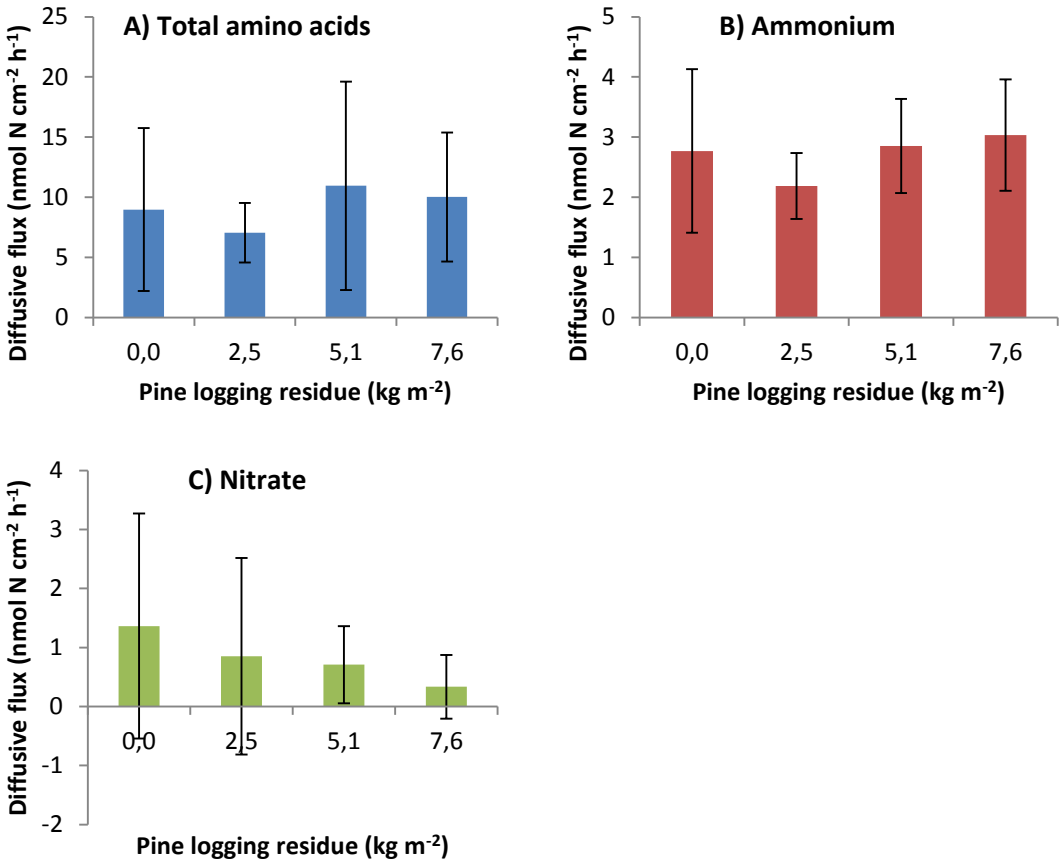

Figure 13. Soil A) total free amino acid, B) ammonium and C) nitrate fluxes in the O_{fh} horizon sampled with microdialysis device *in situ* at the Kiikala logging residue experiment in September 2018. Results are expressed as averages over replicates ± SD (n = 8–12).

Not all of the dialysates were successfully determined for the different N forms due to the small volume of the dialysate sample (260 µl) that did not forgive the mistakes made in sample handling during the analyses. That is why there are different n-values for the different N forms.

7.4 Soil physicochemical properties, N transformations and litter quality

7.4.1 Basic soil characteristics

At the Lapinjärvi study site, soil underneath the spruce logging residue piles had higher pH than soil of the control plots without any logging residues (Table 4; $p < 0.05$). The

control plots at the clear-cut area had lower soil moisture content than the adjacent spruce forest ($p < 0.05$). Also, soil organic matter content was lower at the control plots than underneath the logging residue piles or at the 'MT spruce' site ($p < 0.05$). Soil temperature was significantly higher at the control plots than at the 'MT spruce' site ($p < 0.05$).

Table 4. Soil pH, moisture content, organic matter content, C-to-N ratio and temperature in the O_{fh} horizon at the Lapinjärvi study site in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue. Results are expressed as averages over replicates ± SD (n = 4). For each variable, statistically significant differences between the treatments are indicated with different letters (one-way ANOVA, Tukey's HSD for organic matter, Kruskal-Wallis for pH, moisture, C-to-N ratio and soil temperature, $p < 0.05$).

Site / treatment	pH (H_2O)	Moisture (m-%)	Organic matter (%)	C-to-N ratio	Soil temperature (°C)
Clear-cut (logging residue experiment)					
Control	4.0 ± 0.14^{a}	51 ± 22^{a}	41 ± 10^{a}	29 ± 0.6^{a}	24 ± 1.5^{b}
Spruce LR	4.8 ± 0.24^{b}	80 ± 27^{ab}	59 ± 14^{b}	31 ± 1.9^{a}	21 ± 0.8^{ab}
MT spruce	4.1 ± 0.17^{ab}	86 ± 22^{b}	70 ± 16^{b}	30 ± 2.4^{a}	15 ± 0.9^{a}

At the Kiikala study site, the amount of pine logging residue did not correlate with the soil pH ($r = 0.085$; $n = 12$; p-value of 2-tailed test = 0.792), soil moisture content ($r = 0.189$; $n = 24$; $p = 0.377$), soil organic matter content ($r = 0.373$; $n = 24$; $p = 0.073$), C-to-N ratio ($r = 0.285$; $n = 12$; $p = 0.369$) or soil temperature ($r = 0.269$; $n = 12$; $p = 0.398$) (Table 5).

Table 5. Soil pH, moisture content, organic matter content, C-to-N ratio and temperature in the O_{fh} horizon of the different pine logging residue (LR) levels in Kiikala in September 2018. Results are expressed as averages over replicates ± SD (n = 3).

Pine LR (kg m^{-2})	pH (H_2O)	Moisture (m-%)	Organic matter (%)	C-to-N ratio	Soil temperature (°C)
0.0	3.5 ± 0.13	84 ± 41	71 ± 23	33 ± 2.5	16 ± 1.0
2.5	3.5 ± 0.24	110 ± 35	77 ± 7	35 ± 2.3	16 ± 1.5
5.1	3.3 ± 0.06	74 ± 5	77 ± 18	36 ± 2.1	16 ± 0.6
7.6	3.6 ± 0.24	110 ± 30	86 ± 9	35 ± 3.9	17 ± 0.6

7.4.2 Soil KCl-extractable NH_4^+-N and NO_3^--N and N transformations

Extraction with 1 M KCl showed that even though not statistically significantly in every case, the mean concentrations of NH_4^+-N (Figure 14a) and NO_3^--N (Figure 14b) were higher under the logging residue piles, in comparison to the control plots at the logging residue experiment or to the MT spruce site.

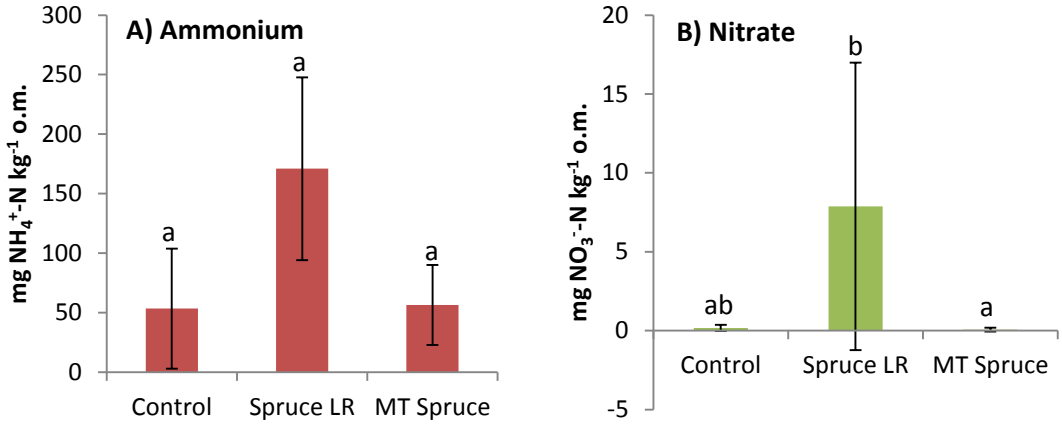

Figure 14. Concentrations of KCl-extractable A) ammonium-N and B) nitrate-N in the O_{fh} horizon of the different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue, o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 4). For each N species, statistically significant differences between the treatments are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

At the Kiikala logging residue experiment, the KCl-extractable NH_4^+-N concentrations showed a slight but not statistically significant decrease with the increasing amount of pine logging residue (Figure 15a; r = -0.329; n = 12; p-value of 2-tailed test = 0.296). Low amounts of nitrate were detected at one pine logging residue level (Figure 15b).

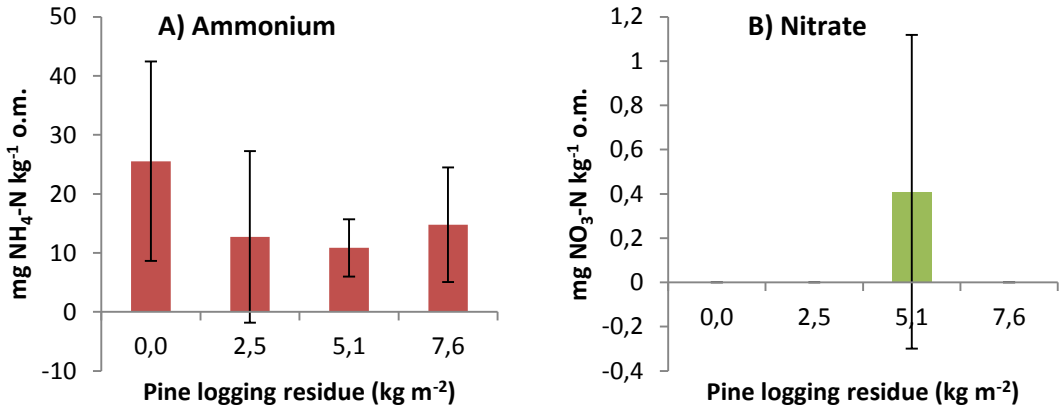

Figure 15. Concentrations of KCl-extractable A) ammonium-N and B) nitrate-N in the O_{fh} horizon of the different pine logging residue levels in Kiikala in September 2018. o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 3).

Incubation experiment showed that the rate of net N mineralization (Figure 16a) and net nitrification (Figure 16b) was substantially higher in the soil underneath the spruce logging residue piles in Lapinjärvi, as compared to the control plots or MT spruce, although the difference was not statistically significant in every case. Net nitrification rate was minute but slightly negative in the MT spruce sample and that is why it differed statistically from the spruce LR, even though the control did not.

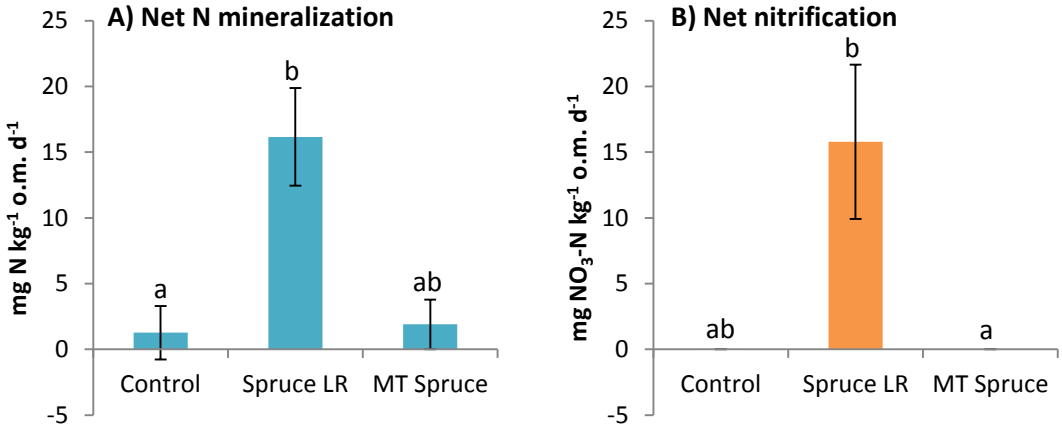

Figure 16. The rates of A) net N mineralization and B) net nitrification in the O_{fh} horizon of the different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue, o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 4). For each N transformation, statistically significant differences between the treatments are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

At the Kiikala logging residue experiment, the net N mineralization rate increased with the amount of pine logging residue (Figure 17; r = 0.624; n = 12; p-value of 2-tailed test < 0.05). Net nitrification was negligible (data not shown).

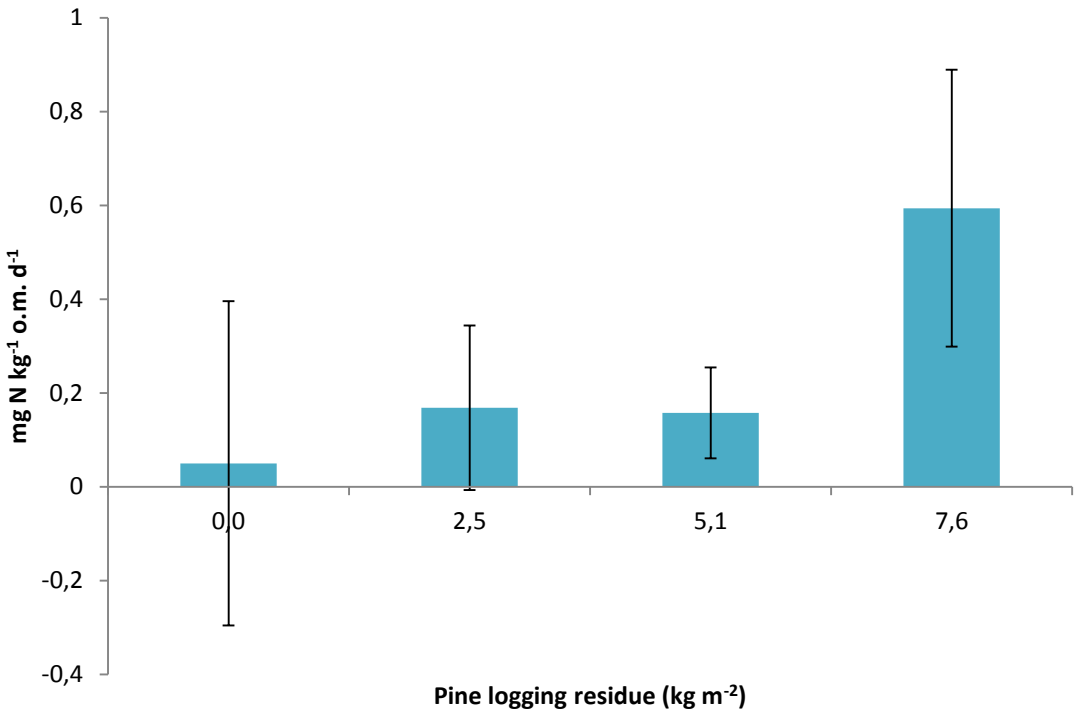

Figure 17. The rate of net N mineralization in the O_{fh} horizon of the different pine logging residue levels in Kiikala in September 2018. o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 3).

7.4.3 Microbial biomass C and N

No significant differences were observed with regard to microbial biomass C and N between the treatments in Lapinjärvi (Table 6). Spruce LR had higher microbial biomass C-to-N ratio than the control but the difference was not statistically significant. The total dissolved nitrogen content was higher under the spruce logging residue piles, as compared to the control plots ($p < 0.05$). Even though not statistically significantly, also the microbial biomass C and the dissolved organic carbon content were higher under the logging residue piles as compared to the control plots or the MT spruce site.

Table 6. Microbial biomass C (C_{mic}) and N (N_{mic}) contents, their ratio (C/N_{mic}), the dissolved organic carbon (DOC) content and the total dissolved nitrogen (TDN) content in the O_{fh} horizon of the different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue, o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 4). For each variable, statistically significant differences between the treatments are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

Site / Treatment	C_{mic} (g kg^{-1} o.m.)	N_{mic} (g kg^{-1} o.m.)	C/N_{mic}	DOC (g kg^{-1} o.m.)	TDN (g kg^{-1} o.m.)
Clear-cut (logging residue experiment)					
Control	8.3 ± 0.6[a]	1.1 ± 0.1[a]	7.6 ± 0.2[ab]	0.7 ± 0.2[a]	0.10 ± 0.10[a]
Spruce LR	9.6 ± 1.9[a]	1.1 ± 0.1[a]	8.7 ± 1.3[b]	2.8 ± 2.1[a]	0.45 ± 0.25[b]
MT spruce	8.5 ± 0.9[a]	1.3 ± 0.2[a]	6.6 ± 0.7[a]	0.8 ± 0.3[a]	0.14 ± 0.11[ab]

The amount of pine logging residue did not correlate with the microbial biomass C ($r = -0.238$; $n = 12$; p-value of 2-tailed test = 0.456) and N contents ($r = 0.112$; $p = 0.730$), their ratio ($r = -0.405$; $p = 0.192$), the dissolved organic carbon content ($r = -0.236$; $p = 0.461$) or the total dissolved nitrogen content ($r = -0.453$; $p = 0.139$) at the Kiikala logging residue experiment (Table 7). The amount of 5.1 kg m^{-2} showed somewhat lower microbial biomass C and N contents than the other logging residue levels.

Table 7. Microbial biomass C (C_{mic}) and N (N_{mic}) contents, their ratio (C/N_{mic}), the dissolved organic carbon (DOC) content and the total dissolved nitrogen (TDN) content in the O_{fh} horizon of the different pine logging residue (LR) levels in Kiikala in September 2018. o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 3).

Pine LR (kg m^{-2})	C_{mic} (g kg^{-1} o.m.)	N_{mic} (g kg^{-1} o.m.)	C/N_{mic}	DOC (g kg^{-1} o.m.)	TDN (g kg^{-1} o.m.)
0.0	7.7 ± 1.3	0.76 ± 0.13	10 ± 0.3	1.07 ± 0.48	0.08 ± 0.01
2.5	7.1 ± 0.7	0.72 ± 0.06	10 ± 0.4	0.63 ± 0.11	0.06 ± 0.01
5.1	6.4 ± 0.3	0.64 ± 0.06	10 ± 1.4	0.70 ± 0.09	0.06 ± 0.01
7.6	7.3 ± 1.2	0.82 ± 0.16	9 ± 1.2	0.84 ± 0.30	0.07 ± 0.01

7.4.4 Volatile monoterpenes in soil atmosphere

At the Lapinjärvi study site, both spruce LR and MT spruce showed clearly higher total concentrations of volatile monoterpenes in soil atmosphere than control, even though only MT spruce differed statistically significantly from control (Figure 18; $p < 0.05$). The dominating volatile monoterpenes were α-pinene, β-pinene and camphene.

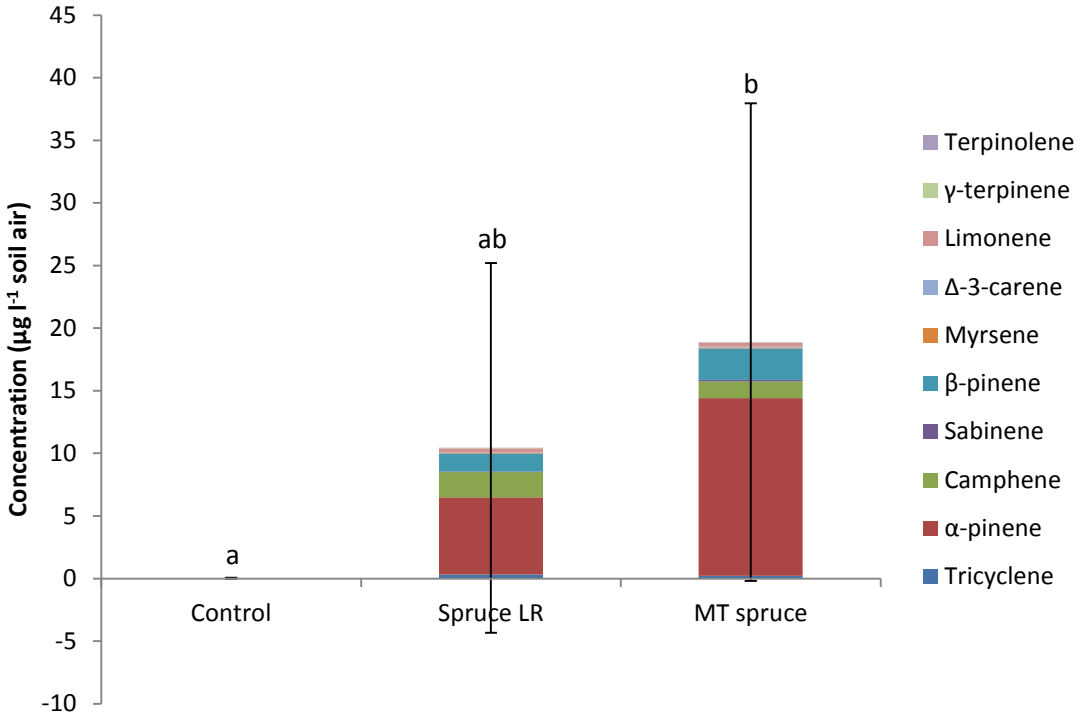

Figure 18. Concentrations of the volatile monoterpenes in soil atmosphere in the O_{fh} horizon of the different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue. Results are expressed as averages over replicates ± SD (n = 4; standard deviations are calculated for the average total volatile monoterpene concentrations). Statistically significant differences between the treatments are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

Kiikala study site (with pine as a main tree species) had higher concentrations of the total volatile monoterpenes in soil atmosphere (Figure 19), as compared to the spruce-dominated Lapinjärvi study site. The amount of pine logging residue and the concentration of the total volatile monoterpenes in soil atmosphere did not show linear

correlation at the Kiikala study site (r = -0.251; n = 12; p-value of 2-tailed test = 0.431). The dominating volatile monoterpenes were α-pinene and Δ-3-carene.

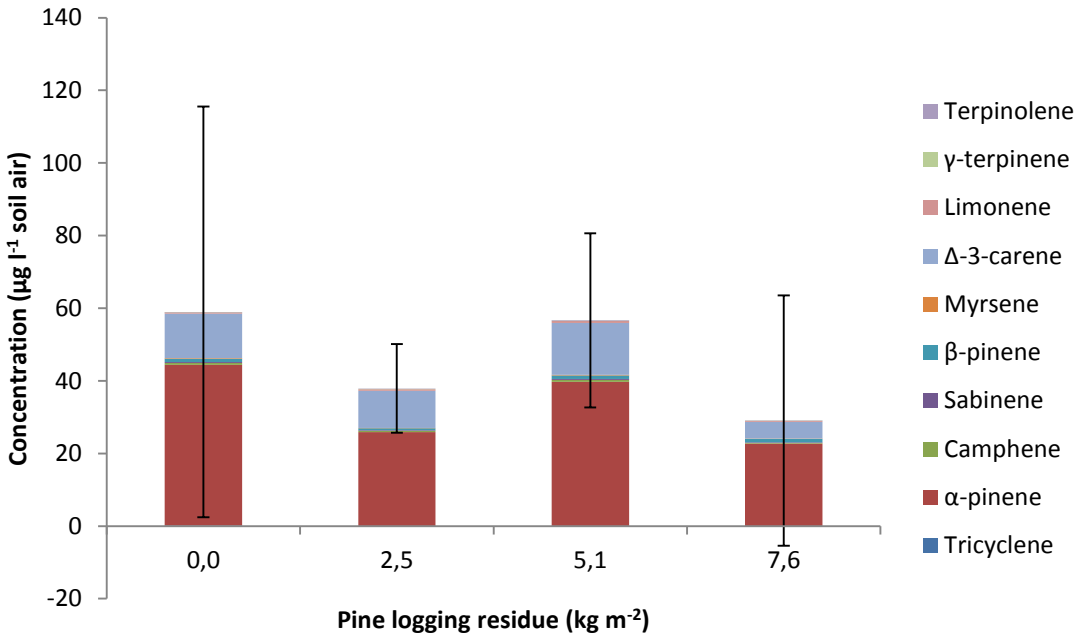

Figure 19. Concentrations of the volatile monoterpenes in soil atmosphere in the O_{fh} horizon of the different pine logging residue levels in Kiikala in September 2018. Results are expressed as averages over replicates ± SD (n = 3; standard deviations are calculated for the average total volatile monoterpene concentrations).

7.4.5 Condensed tannins

In Lapinjärvi, the soil from the MT spruce site had significantly higher condensed tannin concentration than the soil under the spruce logging residue piles or at the control plots (Figure 20; $p < 0.05$).

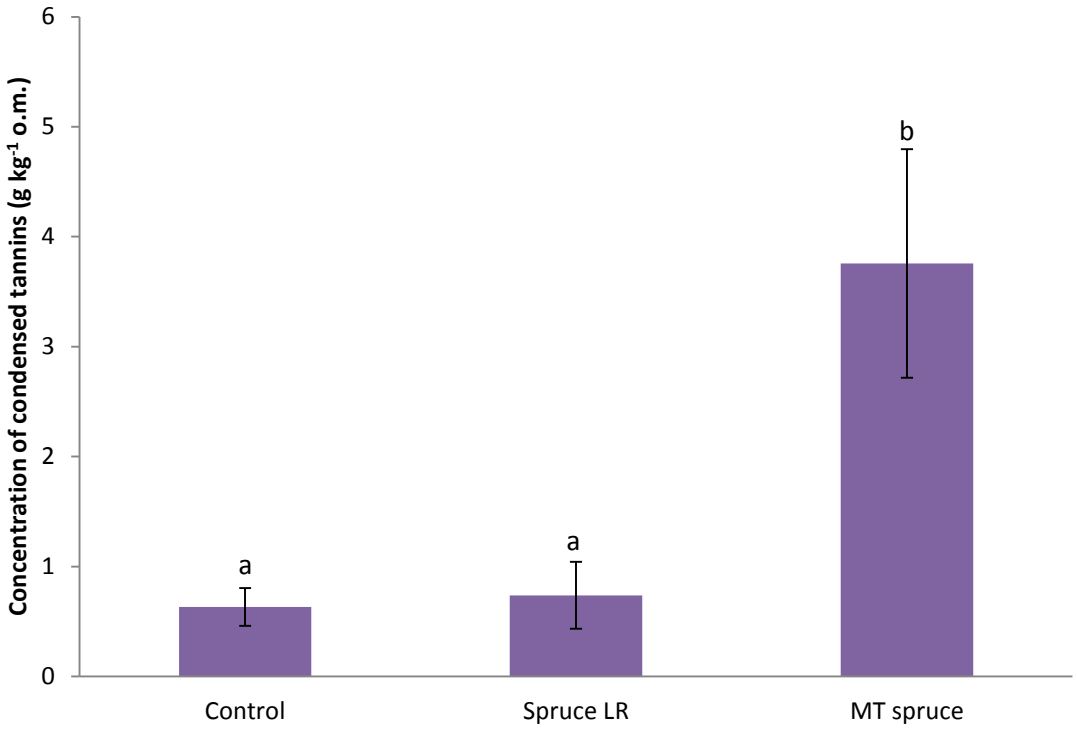

Figure 20. Concentration of the condensed tannins in the O_{fh} horizon of the different treatments in Lapinjärvi in June 2018. Spruce LR = 40 kg m^{-2} of spruce logging residue, o.m. = organic matter. Results are expressed as averages over replicates ± SD (n = 4). Statistically significant differences between the treatments are indicated with different letters (Kruskal-Wallis, $p < 0.05$).

Concentration of the condensed tannins did not correlate with the amount of pine logging residue at the Kiikala study site (Figure 21; r = 0.080; n = 36; p-value of 2-tailed test = 0.642).

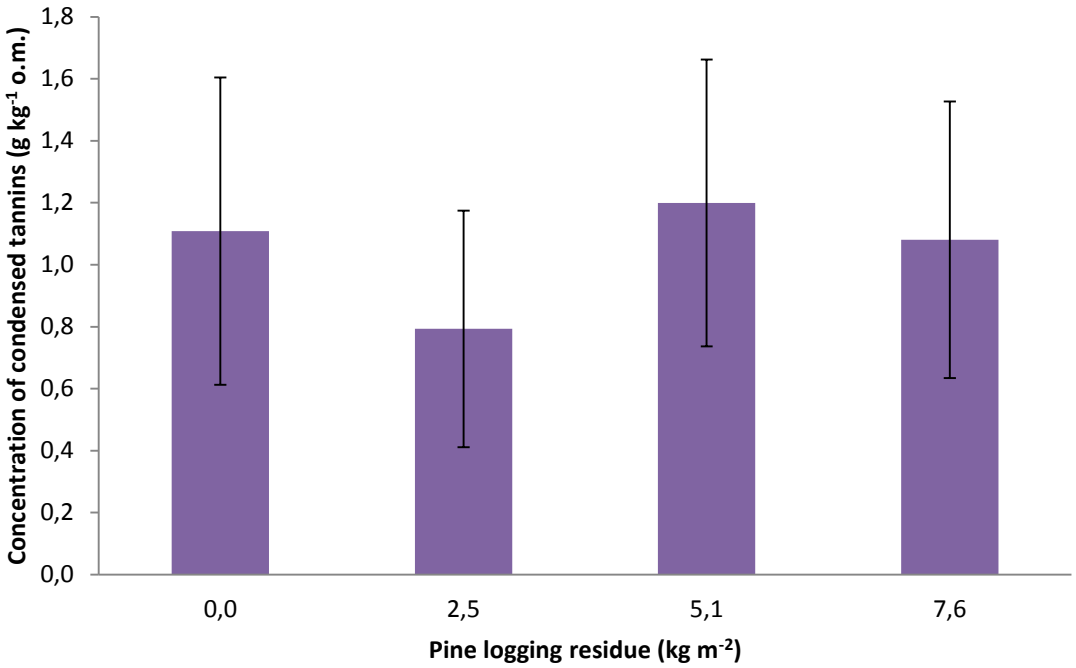

Figure 21. Concentration of the condensed tannins in the O_{fh} horizon of the different pine logging residue levels in Kiikala in September 2018. Results are expressed as averages over replicates ± SD (n = 3).

7.5 Correlations between diffusive N fluxes, soil properties and other factors

In situ soil sampling with the microdialysis device at the Lapinjärvi and Kiikala study sites showed that the diffusive N fluxes of ammonium and the total free amino acids had a statistically significant positive correlation and amino acids had a positive correlation with the total nitrogen flux (Table 8; $p < 0.01$). The diffusive flux of the total free amino acids correlated positively with the dissolved organic carbon and total dissolved nitrogen content ($p < 0.01$). The diffusive ammonium flux showed a positive correlation with the net N mineralization rate ($p < 0.01$). The diffusive NO_3^- flux correlated positively with soil pH and the microbial biomass C and N contents ($p < 0.01$). Moreover, the diffusive NO_3^- flux showed a negative correlation with soil C-to-N ratio, microbial biomass C-to-N ratio and the total volatile monoterpene concentration in soil atmosphere ($p < 0.01$).

Table 8. Pearson correlation coefficients between the diffusive nitrogen fluxes and other studied variables (n = 24 for all variables). Data from Lapinjärvi and Kiikala study sites.

	Diffusive N fluxes (nmol N cm^{-2} h^{-1})			
	Total amino acids	Ammonium	Nitrate	Total N flux
Diffusive N fluxes (nmol N cm^{-2} h^{-1})				
Ammonium	0.550**			
Nitrate	0.015	0.108		
Total N flux	0.983**	0.666**	0.136	
Soil properties				
pH	0.225	0.279	0.639**	0.314
Moisture (m-%)	0.081	0.111	-0.481*	0.040
Organic matter (%)	0.235	0.258	-0.475*	0.202
C-to-N ratio	-0.179	-0.137	-0.609**	-0.246
Temperature (°C)	-0.039	0.119	0.428*	0.030
KCl-extraction and N transformations				
NH_4^+-N (mg kg^{-1} o.m.)	0.427*	0.350	0.503*	0.491*
NO_3^--N (mg kg^{-1} o.m.)	-0.007	0.230	0.310	0.065
Net N mineralization (mg N kg^{-1} o.m. d^{-1})	0.398	0.550**	0.437*	0.491*
Net nitrification (mg NO_3^--N kg^{-1} o.m. d^{-1})	0.354	0.423*	0.384	0.426*
Fumigation-extraction				
Microbial biomass C (g kg^{-1} o.m.)	0.449*	0.252	0.643**	0.509*
Microbial biomass N (g kg^{-1} o.m.)	0.140	0.053	0.761**	0.213
Microbial biomass C-to-N ratio	0.230	0.127	-0.524**	0.171
Dissolved organic C (g kg^{-1} o.m.)	0.538**	0.466*	0.277	0.585**
Total dissolved N (g kg^{-1} o.m.)	0.544**	0.392	0.358	0.587**
Volatile monoterpenes (μg l^{-1} soil air)	0.077	0.086	-0.536**	0.027
Condensed tannins (g kg^{-1} o.m.)	0.017	-0.127	0.275	0.023

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Soil pH correlated positively with the net N mineralization and net nitrification rates (Appendix 3; Table 1; $p < 0.01$). The net N mineralization and nitrification rates were positively correlated with each other and with the KCl-extractable NH_4^+-N concentration (Appendix 3; Table 2; $p < 0.01$). The total concentration of the volatile monoterpenes showed negative correlation with the microbial biomass N content and positive correlation with the microbial biomass C-to-N ratio (Appendix 3; Table 3; $p < 0.01$).

8 DISCUSSION

In this study, soil diffusive ammonium, nitrate and amino acid fluxes were studied *ex situ* at three different soil moisture content levels and after N addition, and *in situ* boreal forest soil with microdialysis. The diffusive N fluxes studied *in situ* at the Lapinjärvi and Kiikala study sites were compared to the soil physicochemical factors, the rates of net N mineralization and net nitrification, microbial biomass C and N contents and the concentrations of the volatile monoterpenes and condensed tannins.

8.1 Relative recoveries of N compounds and microdialysis probe calibration

The 'calibration' procedure of microdialysis probes can be used to characterize the probes (Bungay et al. 1990) as well as to ensure that any damage or fouling of the microdialysis membranes is noticed (Inselsbacher et al. 2011). However, it cannot be linked to describe the *in situ* performance of the microdialysis probes since the resistance caused by the external medium is so much different in the solution versus a heterogeneous medium (Bungay et al. 1990) such as soil. The difference in R_{ext} between soil and a stirred solution makes it problematic to derive the concentrations of the compounds in the soil solution based on their relative recoveries in stirred solution. Despite the given facts, attempts to derive the concentrations of the studied compounds in the soil solution from the dialysate concentrations sampled from soil and the relative recoveries from a stirred solution have been made recently by Hill *et al*. (2019). In this kind of approach, it would be important to consider the physical properties such as moisture content or porosity of a given soil.

The recovery of nitrogen compounds in the dialysate has been improved by using longer, 30 mm membranes (Buckley et al. 2017) in comparison to the 10 mm long membranes (Inselsbacher et al. 2011). The relative recovery of ammonium and nitrate by 10 mm membranes observed in this study showed a similar exponential decrease with the increasing perfusion fluid flow rate to what has been reported previously (Inselsbacher et al. 2011). The calibration of the longer, 30 mm membranes that were used for *in situ* sampling in this study resulted in slightly different values from what has

been found earlier by Buckley *et al*. (2017) for the relative recoveries of ammonium (55 %), nitrate (100 %), and amino acids (40–50 %).

8.2 Diffusive N fluxes *ex situ* and effect of moisture content or N addition

Clear-cut site had lower diffusive amino acid N flux and higher diffusive nitrate flux than the adjacent forest, as expected (hypothesis I). However, these differences were not statistically significant probably because of the small size of data and the large variation in the results. The initial diffusive N fluxes sampled *ex situ* from the sieved soils (clear-cut, MT spruce and VT pine) were dominated by amino acids and their relative share (59–75 %) of the total diffusive N flux was similar to what has been found previously (80 %) by Inselsbacher and Näsholm (2012a) *in situ* boreal forest soil. It must be noted though, that the fluxes of amino acids and ammonium were also 10–20 times higher in this study than those observed by Inselsbacher and Näsholm (2012a) *in situ* (8.3 and 1.5 nmol N cm^{-2} h^{-1} for amino acid N and ammonium, respectively). In addition to differences in the soils studied, one explanation for the difference is that sieving has affected the overall N composition in our soil samples and led to the overestimation of the diffusive fluxes of the total free amino acids and ammonium. The high diffusive flux of the total free amino acid N in sieved soils may have resulted for example from their release from the damaged fine roots and microbes during sieving (Hobbie & Hobbie 2012) and possibly from the depolymerization of proteins into amino acids. Moreover, the high diffusive ammonium fluxes may result from the subsequent mineralization of the released amino acids as Inselsbacher (2014) discussed that sieving can alter soil chemical composition by stimulating microbial degradation of amino acids into inorganic N.

Soil moisture content determines the membrane surface area that is in contact with soil solution. In this study, the diffusive N fluxes were observed at three soil moisture content levels in a laboratory experiment where soil structure was disturbed by sieving. As expected, the diffusive N fluxes were increased with the increasing soil moisture content (hypothesis II) but surprisingly, only the ammonium fluxes showed a response to the increased soil moisture content. The highest diffusive flux of ammonium was observed at moisture content of 60 % WHC that is the optimum moisture for microbial

activity. This indicates that the increase in diffusive NH_4^+ flux may be explained by not only desorption of accumulated NH_4^+ molecules from the soil solid phase into the soil solution but also by the increased mineralization rate. As discussed above, sieving may have altered soil N pools. Moreover, since these were preliminary experiments run in the laboratory while establishing a functional combination of microdialysis and the methods for determination of different N pools, there is some uncertainty in the *ex situ* results. Sample storage at +4 °C after it had been taken from the freezer had affected the fluxes of different N forms, as can be seen by comparing the results of the initial N fluxes after 6 d (Figure 9; MT spruce) or 33 d (Figure 10; 44 % WHC) of storage. The huge decrease in the diffusive flux of the total free amino acid N may be as a result of mineralization that has possibly taken place during the sample storage. The decrease in the diffusive flux of ammonium is probably due to immobilization of it by soil microbes as well as adsorption to soil particles.

By using microdialysis *in situ* temperate forest soil, Leitner *et al*. (2017) found that the rewetting of the dry soil caused a flush of NO_3^- and some neutral hydrophilic amino acids that are considered mobile N forms. Since the *ex situ* experiments of this study were run in laboratory and not in the field, the situation is different because soil sampling, sieving and storage have altered soil N pools, as discussed above. Sample storage has probably affected soil moisture content as well by drying the soil to some extent, and this may have caused the accumulation of ammonium into the bulk soil. Relatively large amount of water was added into the soil sample while adjusting its moisture content to 60 % WHC and this could have been the reason for the mobilization of ammonium that was the major N pool in the soil sample after the long storage.

As hypothesized, nitrogen addition caused a peak in the soil diffusive N fluxes, followed by a subsequent decrease near to the initial state (hypothesis III). Surprisingly, this peak was mostly due to the increase in ammonium fluxes even though the total amount of amino acids added was four times as high as the amount of ammonium. It was expected that the ammonium concentration would return to its initial state during the 2.5 h sampling period as was detected in two soils. In the ‘clear-cut’ soil, however, ammonium flux was still approximately 12 % higher in the end of the sampling period compared to the initial level. The results differed from what Inselsbacher *et al*. (2011)

found for most individual amino acids and nitrate, too – a pulse in the fluxes of all N forms except the amino acids arginine and lysine after N addition. There are several factors that could explain the difference. Inselsbacher *et al*. (2011) used larger amount of N solution (30 ml) that had higher concentration (100 μmol N l^{-1}) and more amino acids (12) in it for a slightly larger amount of soil. In this study, amino acid N added may have been immediately mineralized to ammonium which would explain the high pulse in NH_4^+ flux. However, this contradicts with the finding by Inselsbacher *et al*. (2011) that the microbial degradation during 2 h sampling time is of minor influence whereas adsorption to soil particles and the formation of depletion zone around the microdialysis probes would explain the decrease in N fluxes. Adsorption to soil particles might be a probable explanation for the decrease in the diffusive NH_4^+ flux after the pulse since NH_4^+ can be adsorbed to the negatively charged soil particle surfaces that are abundant in soil colloidal fraction such as clay and organic matter. For proteinaceous compounds, it has been shown that the adsorption mechanisms vary depending on the soil texture and in organic forest floor, the adsorption of added N is quick and high, indicating that organic matter content may be crucial for the adsorption of these compounds (Kanerva et al. 2013).

The results of this study show that the soil moisture content may significantly affect soil N supply rate in the O_{fh} horizon of boreal forest soil. However, this should be further studied with laboratory experiments at several moisture content levels as well as in the field. Especially with regard to the mineral soils, soil structural parameters such as porosity, pore size and their continuity could be investigated since these factors together with soil moisture regulate the flow rate of water in soil that is a key for solute transport. It would be also interesting to couple the measurement of the diffusive N flux to other soil properties such as soil texture and cation exchange capacity since these factors are expected to affect soil N availability as well. After all, these factors were out of the scope of this thesis but it would be advantageous to take these parameters into account in future studies regarding microdialysis as a soil sampling technique.

In this study, there were a couple of uncertainties in the total free amino acid results determined with the OPAME procedure from the dialysates. First, it has been reported by Darrouzet-Nardi *et al*. (2013) that other nitrogen-containing compounds such as

amino sugars and tyramine might interfere with the OPAME procedure and therefore, the result should be reported as total free primary amines. Moreover, it is uncertain how much these compounds contributed to the total free amino acid flux in this study. If they were present in soil solution, it is probable that these small monomers would be found in the dialysate samples as well. Consequently, the total free amino acid results of this study might not exactly reflect only the total free amino acid pool but other primary amines may be included as well.

8.3 Diffusive N fluxes *in situ* boreal forest soil and factors affecting soil N supply

In Lapinjärvi, spruce logging residue piles did not affect statistically significantly the diffusive fluxes of ammonium and nitrate, on the contrary to what was expected (hypothesis IV). An interesting finding was that on average, the total free amino acids contributed least (54 %) to the total N flux in the control plots of the logging residue experiment, as compared to the other treatments in Lapinjärvi or the Kiikala study site and therefore, the relative share of mineral N of the total N flux was highest in these samples. In general, *ex situ* microdialysis sampling showed 2–10 times higher amino acid fluxes and 10–20 times higher ammonium fluxes than the *in situ* microdialysis sampling whereas nitrate fluxes were relatively similar in both cases. The possible reasons for the difference between the results may be due to the differences in sampling, sample storage and pretreatment as has been discussed above (Section 8.2) as well as the fact that the soil moisture content was approximately 2–4 times higher in the soil samples taken for *ex situ* microdialysis than it was during the *in situ* microdialysis sampling.

Total free amino acids contributed most to the total diffusive N flux in the boreal forest soil, as in the previous studies (Inselsbacher & Näsholm 2012a, Oyewole et al. 2016). It must be noted, however, that the results of this study might not be directly comparable to the previous microdialysis work done with 10 mm long membranes (Inselsbacher et al. 2011, Inselsbacher & Näsholm 2012a, Inselsbacher et al. 2014). The longer, 30 mm membranes used in this study had not only substantially higher relative recoveries of the studied compounds but also larger membrane surface area (Buckley et al. 2017). However, as the increased RR and membrane surface area have the opposite effects on

the diffusive flux, the difference in the diffusive fluxes obtained with the 10 mm and 30 mm membranes might not be that large after all. In fact, the diffusive fluxes of the studied N compounds were very similar to those observed by Inselsbacher and Näsholm (2012a) *in situ* boreal forest soil with 10 mm membrane. Differences in the results from this study and Buckley *et al*. (2017) obtained with 30 mm membrane might partly be explained by the differences in the analytical methods of ammonium and amino acids since Buckley *et al*. (2017) used ultra-high performance liquid chromatography instead of the colorimetric indophenol method (NH_4^+) and the fluorometric OPAME procedure (amino acids) used in this study. It was noticed in this study that the longer, 30 mm membranes are quite delicate for the field use and they require careful handling.

The comparison of the diffusive N fluxes and soil KCl-extractable NH_4^+-N and NO_3^--N concentrations resulted in similar conclusion as in previous microdialysis studies (Inselsbacher & Näsholm 2012a, Shaw et al. 2014) - the diffusive fluxes of NH_4^+-N and NO_3^--N are decoupled from their bulk soil concentrations estimated with the KCl extraction. Interestingly, the KCl-extractable NH_4^+-N had a positive correlation with the diffusive fluxes of amino acid N and nitrate that is consistent - given that NH_4^+ is a product of the mineralization of dissolved organic nitrogen and a source material for nitrification.

The KCl-extractable NH_4^+-N concentrations were on the same level as observed by Törmänen *et al*. (2018) in fall 2016 at the Lapinjärvi logging residue experiment. The NO_3^--N concentration observed in this study for the spruce LR treatment, in turn, was only about 5 % of that (160 mg NO_3^--N kg^{-1} o.m.) observed by Törmänen *et al*. (2018). There were no statistically significant differences in the KCl-extractable NH_4^+-N and NO_3^--N between the control plots and the spruce LR treatment observed anymore in June 2018 in this study, unlike in the results by Törmänen *et al*. (2018). The effect of the spruce logging residue on the NH_4^+-N and NO_3^--N concentrations might not be so pronounced anymore after four years since clear-cutting. Lowered NO_3^--N concentrations may result partly from the leaching of NO_3^- from soil, since NO_3^- is an inert solute that is prone to leaching. The increase in microbial biomass C, as compared to its value in 2016 at the spruce LR treatment (Törmänen et al. 2018) may, however,

indicate that part of the NO_3^- loss can be explained with its immobilization by soil microbes.

Soil pH showed positive correlation with the diffusive nitrate flux in the results of this study that is consistent, given that the nitrification rate is higher in a less acidic soil (Paavolainen & Smolander 1998). Net N mineralization and net nitrification rates observed in this study were strongly correlated with the soil pH that is in accordance with the earlier findings by Törmänen *et al*. (2018), explained probably by the stimulated microbial activity due to increased pH (Paavolainen & Smolander 1998). Törmänen *et al*. (2018) showed at the same logging residue experiment in Lapinjärvi that the spruce logging residue may increase soil pH and organic matter content, as compared to the control plots, like was observed in this study as well. The N transformation rates had positive correlation with the microbial biomass C content that is consistent, given that the net release of mineral N is affected by microbial biomass.

In laboratory addition experiments, monoterpenes have been shown to affect soil N cycling (Smolander et al. 2006) but their significance in field conditions is unknown, as reviewed by Smolander *et al*. (2012). The diffusive flux of nitrate showed negative correlation with the concentration of the volatile monoterpenes in this study but no correlation between the volatile monoterpene concentration and the N transformations was found. However, the reduced diffusive flux of nitrate may be partly explained by reduced nitrification rate as indications of the potential of the volatile monoterpenes to inhibit nitrification has been shown in other studies (White 1986, White 1994). The Kiikala study site with pine as main tree species had considerably higher concentration of total volatile monoterpenes in soil atmosphere than the spruce-dominated forest in Lapinjärvi that is in accordance with the previous findings by Smolander *et al*. (2006).

It has been proposed that the condensed tannins could affect litter decomposition in terrestrial environments (Horner et al. 1988) as high-tannin litter is recalcitrant due to for example, the protein-binding effect of tannins (Kraus et al. 2003). The concentration of condensed tannins could therefore be an important indicator of the chemical litter quality in ecological questions that relate to the use and turnover of litter (Gessner & Steiner 2005) and consequently, in questions regarding the role of tannins as mediators

of nutrient availability (Hättenschwiler & Vitousek 2000, Kraus et al. 2003). It was speculated by Smolander *et al*. (2005a) that the higher concentration of condensed tannins might contribute to the lowered rate of net N mineralization. However, no statistically significant correlation between the concentration of condensed tannins and the diffusive N fluxes, net N mineralization or microbial biomass C and N contents was found in this study. The concentration of condensed tannins at the MT spruce site in Lapinjärvi was similar to that observed by Smolander *et al*. (2005a) at a spruce site with comparable soil properties. Compared to the adjacent clear-cut area in Lapinjärvi or the Kiikala pine-dominated study site, the concentration of condensed tannins was substantially higher at the MT spruce site.

9 CONCLUSIONS

The aim of this study was to deepen the knowledge about the soil fine-scale N dynamics by using the recently established microdialysis set-up as a method to study the soil diffusive N fluxes. Results showed that microdialysis is capable not only to monitor the small-scale changes in the diffusive N fluxes but also to possibly help acquire new information about the reaction kinetics at the soil microsites and to help understand the interactions between plant root and soil better. Higher fluxes of ammonium were found in moistened soils, reflecting the moisture-dependency of the diffusive fluxes. The diffusive N fluxes sampled with microdialysis may better reflect the natural state of soil and the ongoing processes than do the traditional extraction methods. It was found that amino acids dominated the total nitrogen flux in the field measurements, and nitrate was detected more frequently from the dialysate samples, as compared to the KCl-extracts. The share of amino acids of the total N flux was lowest at the control plots of the spruce logging residue experiment. Diffusive NH_4^+ flux correlated positively with the net N mineralization rate but no correlation was found between the NH_4^+ flux and the KCl-extractable NH_4^+-N, indicating that the diffusive fluxes may be decoupled from the bulk soil concentrations but instead, they may give valuable information about the soil processes regulating N availability.

In conclusion, microdialysis is an interesting new approach for the studies regarding soil N dynamics since it has a capability to detect small changes in soil N fluxes and the result may reflect N availability in soil microsites. The possibility to study soil N supply instead of the mere bulk soil concentrations may increase the knowledge of the soil processes.

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APPENDIX 1: SUMMARY OF STUDY SITES, TREATMENTS AND ANALYSES

Summary of the study sites, treatments and the analyses. OM = organic matter, DOC = dissolved organic carbon, TDN = total dissolved nitrogen.

	Lapinjärvi						Kiikala			
Sampling time	November 2017			June 2018			September 2018			
Site	Clear-cut	MT spruce	VT pine	Clear-cut (logging residue experiment)		MT spruce	CT pine			
Treatment				Control	Spruce LR		0.0	2.5	5.1	7.6
Basic soil properties (pH, moisture, OM, C-to-N ratio)	x	x	x	x	x	x	x	x	x	x
Ex situ microdialysis	x	x	x							
Moisture		x								
N addition	x	x	x							
In situ microdialysis				x	x	x	x	x	x	x
Soil temperature				x	x	x	x	x	x	x
KCl-extraction and N transformations				x	x	x	x	x	x	x
Microbial biomass C and N, DOC and TDN				x	x	x	x	x	x	x
Volatile monoterpenes				x	x	x	x	x	x	x
Condensed tannins				x	x	x	x	x	x	x

APPENDIX 2: RESULTS FROM STUDY PLOTS DOMINATED WITH MOSS OR SHRUB

Lapinjärvi 2018 samples: MT spruce site - differences in plots with ground vegetation dominated by moss or shrub. DOC = dissolved organic carbon, TDN = total dissolved nitrogen. Results are expressed as averages over replicates ± SD (n = 2 for moss and shrub, n = 4 for MT spruce).

	Moss			Shrub			MT spruce on average		
Diffusive N fluxes (nmol N cm^{-2} h^{-1})									
Total AA	19	±	19	5	±	2	12	±	14
Ammonium	3.4	±	1.2	1.6	±	0.3	2.5	±	1.2
Nitrate	1.7	±	1.0	2.7	±	1.4	2.2	±	1.3
Total N flux	24	±	20	10	±	3	17	±	14
Soil properties									
pH	4.2	±	0.2	4.0	±	0.1	4.1	±	0.2
Moisture (m-%)	74	±	4	99	±	25	86	±	22
Organic matter (%)	59	±	15	81	±	5	70	±	16
C-to-N ratio	29	±	2	31	±	3	30	±	2
Soil temperature (°C)	16.0	±	0.7	14.8	±	0.4	15.4	±	0.9
KCl-extraction and N transformations									
NH_4^+-N (mg kg^{-1} o.m.)	75	±	33	38	±	30	57	±	34
NO_3^--N (mg kg^{-1} o.m.)	0.00			0.12	±	0.17	0.06	±	0.12
Mineralization (mg kg^{-1} o.m. d^{-1})	3.4	±	1.3	0.4	±	0.5	1.9	±	1.9
Nitrification (mg kg^{-1} o.m. d^{-1})	0			0			0		
Fumigation-extraction									
C_{mic} (g kg^{-1} o.m.)	8.7	±	0.7	8.4	±	1.4	8.5	±	0.9
N_{mic} (g kg^{-1} o.m.)	1.4	±	0.1	1.2	±	0.3	1.3	±	0.2
C/N_{mic}	6.4	±	0.8	6.9	±	0.7	6.6	±	0.7
DOC (g kg^{-1} o.m.)	0.91	±	0.39	0.75	±	0.12	0.83	±	0.25
TDN (g kg^{-1} o.m.)	0.19	±	0.17	0.10	±	0.01	0.14	±	0.11
Volatile monoterpenes (μg l^{-1})	23	±	30	15	±	11	19	±	19
Condensed tannins (g kg^{-1} o.m.)	3.0	±	0.6	4.5	±	0.8	3.8	±	1.0

APPENDIX 3: CORRELATIONS BETWEEN STUDIED VARIABLES

Table 1. Pearson correlation coefficients between the soil properties and other studied variables (n = 24 for all variables). Data from Lapinjärvi and Kiikala study sites. DOC = dissolved organic carbon, TDN = total dissolved nitrogen.

	Soil properties				
	pH	Moisture (m-%)	Organic matter (%)	C-to-N ratio	Temperature (°C)
Soil properties					
Moisture (m-%)	-0.133				
Organic matter (%)	-0.432*	0.739**			
C-to-N ratio	-0.810**	0.284	0.571**		
Temperature (°C)	0.465*	-0.389	-0.641**	-0.423*	
KCl extraction and N transformations					
NH_4^+-N (mg kg^{-1} o.m.)	0.801**	-0.029	-0.281	-0.682**	0.436*
NO_3^--N (mg kg^{-1} o.m.)	0.618**	-0.208	-0.218	-0.323	0.187
Mineralization (mg kg^{-1} o.m. d^{-1})	0.813**	-0.004	-0.194	-0.586**	0.382
Nitrification (mg kg^{-1} o.m. d^{-1})	0.741**	0.017	-0.187	-0.512*	0.350
Fumigation-extraction					
C mic (g kg^{-1} o.m.)	0.646**	-0.221	-0.363	-0.695**	0.357
N mic (g kg^{-1} o.m.)	0.700**	-0.300	-0.468*	-0.726**	0.294
C/N mic	-0.524**	0.213	0.417*	0.468*	-0.216
DOC (g kg^{-1} o.m.)	0.519**	0.153	-0.058	-0.383	0.261
TDN (g kg^{-1} o.m.)	0.720**	0.083	-0.147	-0.563**	0.322
Volatile monoterpenes (μg l^{-1})	-0.523**	0.296	0.556**	0.386	-0.427*
Condensed tannins (g kg^{-1} o.m.)	0.014	0.050	0.214	-0.047	-0.471*

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 2. Pearson correlation coefficients between the KCl-extractable NH_4^+-N and NO_3^--N concentrations and N transformations and other studied variables (n = 24 for all variables). Data from Lapinjärvi and Kiikala study sites. DOC = dissolved organic carbon, TDN = total dissolved nitrogen.

	KCl-extraction (mg kg^{-1} o.m.) and		*N transformations (mg N kg^{-1} o.m. d^{-1})*	
	NH_4^+-N	NO_3^--N	Mineralization	Nitrification
KCl-extraction and N transformations				
NO_3^- (mg kg^{-1} o.m.)	0.288			
Mineralization (mg kg^{-1} o.m. d^{-1})	0.919**	0.498*		
Nitrification (mg kg^{-1} o.m. d^{-1})	0.884**	0.479*	0.965**	
Fumigation-extraction				
C mic (g kg^{-1} o.m.)	0.802**	0.060	0.672**	0.644**
N mic (g kg^{-1} o.m.)	0.517**	0.120	0.379	0.268
C/N mic	-0.110	-0.181	-0.048	0.081
DOC (g kg^{-1} o.m.)	0.823**	0.114	0.818**	0.855**
TDN (g kg^{-1} o.m.)	0.962**	0.251	0.916**	0.907**
Volatile monoterpenes (μg l^{-1})	-0.333	-0.186	-0.288	-0.260
Condensed tannins (g kg^{-1} o.m.)	-0.142	-0.182	-0.203	-0.235

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).

Table 3. Pearson correlation coefficients between the microbial biomass C and N contents, their ratio, dissolved organic carbon (DOC) content, total dissolved nitrogen (TDN) content and the total concentration of volatile monoterpenes in soil atmosphere (n = 24 for all variables). Data from Lapinjärvi and Kiikala study sites.

	Fumigation-extraction					
	C mic (g kg^{-1} o.m.)	N mic (g kg^{-1} o.m.)	C/N mic	DOC (g kg^{-1} o.m.)	TDN (g kg^{-1} o.m.)	Volatile monoterpenes (μg l^{-1})
Fumigation-extraction						
N mic (g kg^{-1} o.m.)	0.725**					
C/N mic	-0.213	-0.807**				
DOC (g kg^{-1} o.m.)	0.744**	0.255	0.194			
TDN (g kg^{-1} o.m.)	0.768**	0.394	0.022	0.903**		
Volatile monoterpenes (μg l^{-1})	-0.486*	-0.644**	0.601**	-0.204	-0.242	
Condensed tannins (g kg^{-1} o.m.)	0.060	0.384	-0.399	-0.139	-0.141	-0.025

**. Correlation is significant at the 0.01 level (2-tailed).
*. Correlation is significant at the 0.05 level (2-tailed).